University of Alberta

NORADRENERGIC AND OPIOIDERGIC REGULATION OF GONADOTROPIN SECRETION IN THE FEMALE PIG

by

HEATHER JUNE WILLIS ©

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NORADRENERGIC AND OPIOIDERGIC REGULATION OF GONADOTROPIN SECRETION IN THE FEMALE PIG

submitted by

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in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY in ANIMAL PHYSIOLOGY

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ABSTRACT

Endogenous opioid peptides (EOP's) inhibit GnRH and LH secretion during lactation in the sow, however, EOP inhibition is not apparent until 78h post-partum. The first experiment was designed to determine if the EOP's function in late gestation, immediately prior to a time when the EOP's do not inhibit LH secretion. On either d107 or d108 of gestation, sows were given the opioid antagonist, naloxone; on the alternate day, the sows acted as their own controls. Naloxone increased LH ($P<0.03$) and decreased prolactin ($P>0.015$) secretion. LH and prolactin secretion were positively correlated during the control periods, and a daily rhythm was detected in prolactin secretion.

Opioidergic/noradrenergic interactions regulating GnRH secretion were examined in a second experiment, to determine if the EOP inhibit GnRH secretion pre-synaptically, at the noradrenergic neuron, or post-synaptically, at the GnRH neuron. On d7, 9 and 11 of lactation, sows received 3 treatments in randomized order; naloxone (NAL), phenylephrine (PHEN), an α_1-noradrenergic agonist, or NAL/PHEN combined. Treatment with NAL and NAL/PHEN increased LH secretion compared to treatment with PHEN alone ($P<0.02$). After weaning, 2 sow groups received either, morphine or morphine and PHEN combined; a third control group received no treatment. Morphine suppressed LH secretion compared with the controls ($P<0.04$). In lactation and after weaning, PHEN was unable to overcome opioidergic inhibition of LH secretion, indicating that the EOP's directly inhibit GnRH neurons or that intravenous PHEN was ineffective.

In light of problems associated with peripheral administration of drugs to elicit a central response, an *in vitro* perfusion and assay system were developed in a series of preliminary studies. In a subsequent experiment, GnRH secretory responses to various

noradrenergic drugs were measured to determine whether the noradrenergic system is involved in GnRH pulse generation in the gilt. Using PHEN and the α_1-noradrenergic antagonist, prazosin, it was determined that an α_1-noradrenergic mechanism stimulates GnRH secretion in the porcine hypothalamus.

In a final experiment, opioidergic/noradrenergic interactions were examined using an *in vitro* approach. The highest dose of NAL increased (P=0.001), and PHEN at all doses had no effect on (P=0.11), GnRH secretion from hypothalamic tissue obtained from sows during early gestation, indicating that the EOP's inhibit GnRH secretion post-synaptically, at the level of the GnRH neuron. These results demonstrate that opioids are an important inhibitory component, and that an α_1-noradrenergic receptor mediated mechanism is a stimulatory component of the GnRH pulse generator in the female pig.

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LIST OF ABBREVIATIONS

^3H	tritium
5-ADMP	5-amino-2,4-dihydroxy-α-methylphenylethylamine (specific noradrenergic neurotoxin)
5-HT	serotonin
5,7-DHT	serotonin neurotoxin
6-OHDA	6-hydroxydopamine (catecholamine synthesis inhibitor)
α	alpha (noradrenergic receptor class)
αMPT	α-methyl-p-tyrosine (noradrenergic synthesis inhibitor)
β	beta (noradrenergic receptor class)
δ	delta (opioid receptor class)
γ	gamma (ie. gamma aminobutyric acid)
κ	kappa (opioid receptor class)
μ	mu (opioid receptor class)
AIMAX	N-methyl-N^{-1}[1-methyl-2-propenyl]1,2 hydrazine-dicarbothioamide (noradrenergic synthesis inhibitor)
ARC	arcuate nucleus
BAC	baclofen ($GABA_B$ receptor agonist)
BIC	bicuculline ($GABA_A$ receptor antagonist)
Ca^{2-}	calcium ion
CB-154	bromocriptine (dopamine receptor agonist)
CL	corpus luteum
CRH	corticotropin releasing hormone
DDC	diethyldithiocarbamate (dopamine-ß-hydroxylase inhibitor)
EAA	excitatory amino acid
EOP	endogenous opioid peptide
DA	dopamine

GABA	gamma aminobutyric acid
GnRH	gonadotropin releasing hormone
HAL	haloperidol (DA_2-receptor antagonist)
HST	hypophysial stalk transection
HT	hypothalamic transected
i.c.v.	intracerebroventricular
ISO	isoproterenol (ß-noradrenergic receptor agonist)
i.v.	intravenously
LH	luteinizing hormone
MB	mamillary bodies
MBH	medio basal hypothalamus
ME	median eminence
MPOA	medial preoptic area
MUA	multi unit activity
MUS	muscimol ($GABA_A$ receptor agonist)
NAL	naloxone (opioid receptor antagonist)
NE	norepinephrine
NPY	neuropeptide Y
OC	optic chiasm
OVX	ovariectomized
PBZ	phenoxybenzamine (α-noradrenergic receptor antagonist)
PHEN	phenylephrine (α_1-noradrenergic receptor agonist)
PO/AH	preoptic anterior hypothalamic
POMC	proopiomelanocortin
PRL	prolactin
PRAZ	prazosin (α_1-noradrenergic receptor antagonist)
PROP	propranolol (ß-noradrenergic receptor antagonist)
V	third ventricle

CHAPTER 1
INTRODUCTION

GnRH release, and the subsequent release of LH, are both key elements of reproduction in the mammal. In the absence of pulsatile GnRH, whether this occurs naturally, for example during lactational anestrus (Cox and Britt, 1982; Britt *et al.*, 1985; Rojanasthien *et al.*, 1987 and 1988, De Rensis *et al.*, 1991), or is experimentally induced (Esbenshade and Britt, 1985; Esbenshade *et al.*, 1986; Kraeling *et al.*, 1986; Esbenshade, 1991), pulsatile LH secretion and ovarian follicular development cease. In the swine industry, productivity and profit are based on the number of pigs produced per sow per year, and reproductive efficiency is therefore a prime concern. Reproductive efficiency is affected by many factors such as age at puberty, conception rate, ovulation rate, embryo survival, farrowing rate, weaning percentages and the number of unproductive days a sow has per year. As gestation length is fixed, unproductive days in the sow are based on lactation length and the weaning to estrus interval. Therefore, the occurrence of lactational anestrus in the sow, and the mechanisms involved, are of great importance to the industry. In many other species, the central control of GnRH/LH secretion has been studied in depth, and many neuroendocrine factors have been identified as playing an important role in LH pulse modulation. However, in the pig, there is an overall lack of information regarding the central regulation of GnRH, and thus LH secretion.

The literature review presented in Chapter 2 of this thesis, describes many of the studies which have been involved in the identification of neural regulatory systems in other species. Where appropriate, when data have been available for the pig, entire sections have been devoted to the central regulation of GnRH secretion in the pig. Included in this review are studies regarding neurotransmitters and concepts which I believe are important to GnRH secretion, overall, and those which I believe may be important to the regulation of GnRH secretion in the female pig. Although the studies described in this thesis are not of an applied nature, the data from which could be used directly at the farm level, they do lead to a better understanding of the central regulatory systems governing reproduction. The more knowledge that can be accumulated, the better we are able to manipulate these systems and improve the clinical aspects of swine reproduction and fertility.

With these ideals in mind, a series of *in vivo* experiments were planned to identify the hypothalamic systems which are involved in the regulation of GnRH secretion in the sow during lactation, and how they may be manipulated to induce a fertile estrus during lactation or to reduce the weaning to estrus interval, thereby reducing the number of unproductive days in the year. Our laboratory has previously confirmed that suckling induced inhibition of LH secretion is mediated by the endogenous opioid peptides (EOP) during established lactation in the sow (De Rensis *et al.*, 1993a). However, it has also been demonstrated that this opioidergic regulation does not appear to mediate the initial suckling induced inhibition of LH secretion which occurs prior to 78 hours post-partum (De Rensis, 1993; De Rensis *et al.*, 1993a). Therefore, the first experiment was designed (Chapter 3) to determine if the EOP regulate LH secretion in late gestation, a period we felt was immediately prior to the period when the EOP could not be antagonized, and exogenous opioids also could not inhibit LH

secretion. The experiment presented in Chapter 3 is an extended version of a paper accepted by 'Biology of Reproduction' (55:318-324, 1996).

Although the role for an opioidergic mechanism which inhibits LH secretion during lactation has been well established (Barb *et al.*, 1986a; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988; De Rensis, 1993; De Rensis *et al.* 1993), how the opioids mediated their effects on the GnRH system still had to be established. A second experiment, described in Chapter 4, was designed to determine whether the EOP inhibit GnRH secretion at a level higher than the GnRH neuron, via inhibition of a stimulatory noradrenergic system, or post-synaptically, directly on the GnRH neurons themselves. This experiment was completed in two parts, the first during established lactation, when the EOP are inhibitory to GnRH secretion. A noradrenergic agonist was administered to determine whether exogenous norepinephrine (NE) could overcome the endogenous opioid blockade on GnRH secretion. If LH secretion increased in response to the exogenous noradrenergic stimulus, we hypothesized that the inhibition is via the noradrenergic system because no amount of exogenous stimulus would increase LH secretion if the opioidergic block was directly on the GnRH neuron. In the second part of this experiment, after weaning the EOP no longer inhibit GnRH secretion (Armstrong *et al.*, 1988), so opioid tone was replaced using morphine to suppress GnRH concentrations. Again the NE agonist was administered to determine if an exogenous noradrenergic stimulus could overcome an opioidergic block on GnRH secretion. The results from this study would indicate whether the same systems were still involved in the regulation of GnRH secretion in the absence of the suckling stimulus.

The *in vivo* approaches previously used in Chapters 3 and 4 used the quantification of LH in plasma as an indicator of central GnRH secretion but were potentially limited with regard to those drugs that could be assumed to cross the blood-brain barrier, even when given at pharmacological doses intravenously. This prompted consideration of an alternative approach to studying the neuroendocrine regulation of GnRH secretion in the sow. Barb *et al.* (1994) have recently successfully used an *in vitro* perfusion of porcine medial preoptic area tissue to further describe opioidergic regulation of GnRH secretion at the hypothalamic level. The results of a series of preliminary studies to develop an *in vitro* perfusion system and GnRH assay for quantifying GnRH from porcine hypothalamic tissues are included in Appendix 1. In subsequent experiments described in Chapters 5 and 6 of this thesis, hypothalamic tissue was collected from follicular phase gilts and sows during early gestation, to attempt to describe the noradrenergic and opioidergic regulation of GnRH secretion directly at the hypothalamic level. From studies in other species (Leung *et al.*, 1982; Kaufman *et al.*, 1985; Clough *et al.*, 1988) it is evident that during the follicular phase of the estrous cycle or when ovariectomized animals are treated with estradiol, NE is an important stimulatory component of the GnRH pulse generator. Parvizi and Ellendorff (1978 and 1982), and more recently Chang *et al.* (1993), have implicated the noradrenergic system in the regulation of pulsatile LH secretion in the pig. Chapter 5 describes the *in vitro* perfusion of tissue from follicular phase gilts and the GnRH responses to treatment with various noradrenergic agonists and antagonists, to determine if the noradrenergic system is involved in GnRH pulse generation in the pig, and if it is, via which receptor type does it function. Finally, Chapter 6 readdressed the hypothesis proposed in Chapter 4, using a different

physiological model. As has been demonstrated previously, the EOP are inhibitory to GnRH secretion during periods in which circulating progesterone concentrations are high (Barb *et al.*, 1986b; Szafranska *et al.*, 1994). Therefore, we asked whether the EOP inhibit GnRH secretion during progesterone dominated environment of early gestation, and if they do, is it via a pre-synaptic, at the noradrenergic neuron, or post-synaptic level, at the GnRH neuron, within the hypothalamus.

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CHAPTER 2
LITERATURE REVIEW

2.1 Importance of GnRH to Reproduction in the Female Pig

Gonadotropin releasing hormone (GnRH) is a decapeptide released from neurons in the hypothalamus. In the pig, GnRH perikarya are located in the medial preoptic area (MPOA) of the hypothalamus; axons course through the lateral hypothalamus and along the walls of the third ventricle, terminating in the median eminence at the hypophysial portal vessels (Kineman *et al.*, 1988). Pulsatile release of GnRH into the portal vessels stimulates pulsatile luteinizing hormone (LH) secretion from the anterior pituitary (Leshin *et al.*, 1992a). When GnRH release is impaired, LH release is also similarly affected. When this occurs, reproductive function in the sow or gilt fails, for without GnRH there can be no ovarian follicular development or subsequent increases in circulating estradiol, and consequently, the estradiol and LH surges which promote ovulation, fail to occur.

The importance of GnRH to overall reproductive success has been demonstrated in studies which use GnRH replacement to stimulate LH secretion during physiological states in which endogenous GnRH secretion is absent. Hourly intravenous (i.v.) administration of GnRH, to mimic endogenous GnRH pulsatility, induced follicular development, estrus and ovulation in lactating sows (Cox and Britt, 1982a; Britt *et al.*, 1985; Rojanasthien *et al.*, 1987 and 1988; Rojkittikhun *et al.*, 1991a), and weaned anestrous sows (Armstrong and Britt, 1985). De Rensis *et al.* (1991) found that administering pulsatile GnRH every two hours to lactating sows increased LH secretion and follicular development, as measured by follicular diameter and estradiol concentration in follicular fluid.

Immunoneutralization of GnRH has also been used to demonstrate that the absence of endogenous GnRH secretion prevents normal reproductive function. After immunoneutralization of GnRH in ovariectomized (OVX) and follicular phase gilts, which resulted in suppressed LH secretion, administration of pulsatile exogenous GnRH restored LH pulsatility (Esbenshade and Britt, 1985; Esbenshade *et al.*, 1986; Esbenshade, 1991). More recently, Chang *et al.* (1993a) passively immunized progesterone-treated OVX gilts against GnRH and thereby decreased LH responses to naloxone (NAL) treatment, but GnRH challenges resulted in LH release from the pituitary.

The permanent disruption of the pituitary portal vessels by hypophysial stalk transection (HST) also results in a cessation of LH secretion, and therefore, impairs reproductive function. However, pulsatile administration of exogenous GnRH in these animals again restores LH pulsatility (Molina *et al.*, 1986; Kesner *et al.*, 1989a; Kraeling *et al.*, 1990). Hourly pulses of GnRH administered to HST prepubertal gilts, pretreated with pregnant mare serum gonadotropin (Kraeling *et al.*, 1990), and to unmodified prepubertal gilts (Carpenter and Anderson, 1985; Lutz *et al.*, 1985; Pressing *et al.*, 1992) induced estrus and ovulation. These experiments all underline the importance of pulsatile GnRH secretion from the hypothalamus for reproductive function in the pig.

2.2 Regulation of Gonadotropin Secretion in the Female Pig

2.2.1 Lactational Anestrus

Most mammals undergo a period of lactational amenorrhea or anestrus while suckling their young. Uterine involution occurs during this period and the dam is able to devote attention and metabolic resources to rearing the current litter. Although suckling is the oldest form of birth control, it is only relatively recently that we have understood the mechanisms involved in the suppression of follicular growth and development during lactation. Originally, the interactions between LH and PRL secretion suggested that hyperprolactinemia might inhibit LH secretion during lactation in the sow (Booman *et al.*, 1982; Kraeling *et al.*, 1982; Bevers *et al.*, 1983; Dusza *et al.*, 1984; Mattioli and Seren, 1985). However, the results of a number of studies were inconclusive in establishing this effect and several groups have since demonstrated that endogenous opioid peptides (EOP) are involved in the suckling mediated inhibition of LH secretion during lactation in the sow (Barb *et al.*, 1986b; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a; De Rensis *et al.*, 1993a).

If one considers species other than the pig, there is also evidence for an opioid component to suckling induced inhibition of LH (Sirinathsinghji and Martini, 1984 (rat); Gregg *et al.*, 1986 (sheep); Whisnant *et al.*, 1986 (cattle)). However, there also appear to be other factors involved in the regulation of lactational anestrus. In ruminant species, particularly the sheep, there appears to be a strong steroidal feedback component to the initial post-partum inhibition of LH secretion (Gregg *et al.*, 1986 (sheep); Rund *et al.*, 1989 (cow)). Also in sheep, seasonality confounds rebreeding, as lambing season normally coincides with the start of the non-breeding season (Pope *et al.*, 1989). It has also been shown that in cattle, olfaction, visual cues and maternal behaviour, as well as the suckling stimulus, play an important role in causing lactational anestrus (Zalesky *et al.*, 1990; Silveira *et al.*, 1993; Williams *et al.*, 1993; Griffith and Williams, 1996; reviewed by Williams *et al.*, 1996). Finally, in rats (Smith, 1978; Sirinathsinghji and Martini, 1984), primates (Gordon *et al.*, 1992) and humans (Nunley *et al.*, 1991) hyperprolactinemia does appear to be important in the suppression of LH secretion during lactation.

2.2.2 Luteinizing Hormone - Estrous Cycle

As previously discussed, GnRH is the gonadotropin releasing factor in the hypothalamus of the pig, and without it, LH secretion from the pituitary does not occur unless GnRH is replaced (Esbenshade and Britt, 1985; Esbenshade *et al.*, 1986; Molina *et al.*, 1986; Kesner *et al.*, 1989a; Kraeling *et al.*, 1990; Esbenshade, 1991). Leshin *et al.* (1992a) has also shown a temporal relationship between GnRH pulses reaching the anterior pituitary and peripheral pulsatile LH concentrations. It was determined that every LH pulse was preceded by a GnRH pulse, but that not every GnRH pulse resulted in an LH pulse in OVX gilts. The authors suggest that these silent GnRH episodes are required to maintain pituitary responsiveness to subsequent GnRH stimulation.

In the sow or gilt, the gonadal steroids, estrogen and progesterone are important regulators of reproductive function, and determine the pattern of LH secretion; high

amplitude, low frequency pulses occur during the luteal phase of the cycle (progesterone dominant; days 1-15/16, standing heat designated as day 0) (Parvizi *et al.*, 1976; Van de Wiel *et al.*, 1981), whereas a lower amplitude, higher frequency pulsatile pattern of secretion is seen in the follicular phase (estrogen dominant; days 16-21) and during the pre-ovulatory surge of LH (day 21/1) (Van de Wiel *et al.*, 1981). At tonic levels, LH acts with FSH to induce follicle development and eventual estradiol secretion from large preovulatory follicles in the cycling animal (as reviewed by Foxcroft *et al.*, 1994).

Administration of estrogen to OVX or weaned sows (Stevenson *et al.*, 1981; Cox and Britt, 1982b; Edwards and Foxcroft, 1983a; Britt *et al.*, 1991) results in a biphasic pattern of LH secretion. A period of negative feedback during which LH secretion is inhibited by estradiol is followed by a period of positive feedback during which LH secretion is stimulated. Positive feedback finally culminates in an LH surge 50-55 hours after estradiol administration (Edwards and Foxcroft, 1983a and 1983b), and approximately 40 hours following the LH surge, ovulation occurs (Van de Wiel *et al.*, 1981). During the period of negative feedback, LH secretion is characterized by decreased amplitude and frequency of LH pulses, and a decreased pituitary sensitivity to GnRH (Kesner *et al.*, 1987). In the pig, pulsatile LH secretion is low for approximately 36 to 52 hours prior to the LH surge induced by estradiol benzoate in OVX gilts (Cox and Britt, 1982b; Kesner *et al.*, 1987). This period of suppressed GnRH/LH release is considered necessary for the estradiol induced surge of LH to occur. The estradiol induced surge of LH in OVX gilts was greatly diminished or completely blocked when exogenous pulsatile GnRH was continued throughout the period of negative feedback, compared with those gilts receiving GnRH during the positive feedback period (Kesner *et al.*, 1989b).

Estradiol positive feedback occurs after the period of negative feedback, and is the period in which a preovulatory estradiol surge induces a large release of GnRH from the hypothalamus, resulting in an LH surge and ovulation (Elsaesser *et al.*, 1978; Dial *et al.*, 1983). It is marked by an increase in frequency of GnRH release from the hypothalamus, an increased pituitary responsiveness to GnRH and increased LH pulsatility (Kesner *et al.*, 1989b; Britt *et al.*, 1991). Depending on the species, estradiol functions at both the hypothalamus and the pituitary to induce the LH surge, 1) to release massive quantities of GnRH into the portal vessels and, 2) to directly sensitize the pituitary to GnRH by increasing GnRH receptor number and increasing rate of LH synthesis (Aiyer *et al.*, 1974 (rat); Nett *et al.*, 1984; Caraty *et al.*, 1989 (Sheep)).

As previously mentioned, in other species, estradiol sensitizes the pituitary to GnRH secretion during the positive feedback phase. In the pig, estradiol appears to decrease the responsiveness of gonadotropes to GnRH for a period of 12 to 20 hours, a negative feedback effect on the pituitary (Cox and Britt, 1982b; Kesner *et al.*, 1987). In OVX gilts which had been immunized against native GnRH, Britt *et al.* (1991) found that the pattern of GnRH administration during positive feedback can influence the amplitude, but not the duration, of the LH surge, and that estrogen decreases the pituitary's responsiveness to GnRH. This model demonstrates that initial negative feedback for 6 to 12 hours may be mediated by decreased pituitary responsiveness, but the remaining period of negative feedback results from estradiol induced suppression of GnRH secretion at the hypothalamic level. Together, these

mechanisms allow for the accumulation of sufficient LH within the gonadotropes to maintain the preovulatory LH surge (Britt *et al.*, 1991).

The mechanism by which estradiol exerts its effect on hypothalamic GnRH neurons is not clear. It is unlikely to be a direct effect, as immunocytochemical studies in other species have shown that GnRH neurons do not have estrogen receptors (Watson *et al.*, 1992; Herbison *et al.*, 1992; Lehman and Karsch, 1993; Herbison *et al.*, 1995). Other hypothalamic neurons which contain estrogen receptors must therefore mediate the effects of estrogen negative and positive feedback on GnRH neurons. It is possible that positive feedback mechanisms involve the removal of negative feedback influences, as well as the involvement of estrogen sensitive stimulatory neurons. Estrogen accumulating neurons, which are possible candidates for the mediators of hypothalamic effects of estradiol, based on evidence in other species, are norepinephrine (Heritage *et al.*, 1977), GABAergic (Herbison *et al.*, 1993) and ß-endorphin (Lehman and Karsch, 1993) type neurons. Opioidergic neurons have also been shown to possess functional progesterone receptors (Bethea and Widmann, 1996), and are therefore prime candidates for the mediation of progesterone negative feedback during the luteal phase of the estrous cycle.

Progesterone secretion in the luteal phase of the estrous cycle (days 1-15/16) shows a distinct rise in peripheral circulation by day 3 or 4 of the cycle, following luteinization of the follicles after ovulation (Tillson and Erb, 1967; Parvizi *et al.*, 1976; Van de Wiel *et al.*, 1981). During this period of the cycle, LH acts as a luteotropic signal to maintain the corpus luteum (CL) and stimulate progesterone secretion (Cook *et al.*, 1967; Parvizi *et al.*, 1976). Parvizi *et al.* (1976) found that during the luteal phase, each progesterone peak was preceded by an LH episode. Peripheral progesterone concentrations decline by approximately day 15 or 16 of the cycle, following luteolysis of the CL in response to uterine prostaglandin $F_{2\alpha}$ (Tillson and Erb, 1967; Parvizi *et al.*, 1976; Guthrie *et al.*, 1979; Van de Wiel *et al.*, 1981). During the luteal phase, LH secretion is typified by large amplitude, low frequency pulsatile secretion, and mean concentrations of less than 1 ng/ml (Parvizi *et al.*, 1976; Van de Wiel *et al.*, 1981; Ziecik *et al.*, 1982). This pattern of secretion is due to the effects of progesterone negative feedback on GnRH/LH secretion. These effects have been shown to be mediated by the EOP during the luteal phase in the pig (Barb *et al.*, 1985 and 1986a; Kesner *et al.*, 1986; Chang *et al.*, 1993a), and will be discussed in more depth later in this review.

2.2.3 Luteinizing Hormone - Gestation

During gestation in the pig, LH functions as a luteotropin, supporting the corpus luteum (CL) of pregnancy. Thus in pregnant gilts, immunization against LH resulted in CL regression and abortion (Spies *et al.*, 1967). Jammes *et al.* (1985) showed that there was a dramatic increase in LH binding on luteal membranes during the first 10 days of gestation; binding then remained constant until a further increase at day 50. *In vitro* studies have shown that LH stimulates progesterone secretion from luteal cells collected in early gestation (Cook *et al.*, 1967; Wiesak, 1985).

LH is secreted in a pulsatile manner throughout gestation (Parvizi *et al.*, 1976; Ziecik *et al.*, 1982; Ziecik *et al.*, 1982/1983; Smith and Almond, 1991). Ziecik *et al.* (1982) showed

that the frequency of episodic LH secretion during the first 14 days of pregnancy (based on an average gestation of 114/115 days) was high and variable, between 1.2-2.1 ng/ml, but declined through day 24 to 0.4-0.6 ng/ml, concentrations typical of luteal phase. The authors suggest that the high levels of LH during the first 14 days may have been stimulated by coitus, and that this pattern of LH secretion likely represents the establishment of the CL of pregnancy and then the subsequent maintenance of the CL.

Three weeks prior to parturition, LH concentrations were found to be low and progesterone concentrations were high (Parvizi *et al.*, 1976; Ziecik *et al.*, 1982; Kraeling *et al.*, 1992a; Szafranska *et al.*, 1994). Furthermore, in a 12-hour period, three of four LH episodes were followed by increases in progesterone secretion. However, this relationship was abolished by 41 hours prior to parturition (Parvizi *et al.*, 1976). Szafranska *et al.* (1994) have shown that LH secretion is regulated by the EOP in mid-gestation in the sow. These results indicate that LH secretion during gestation in the sow is responsible for maintenance of the CL and progesterone secretion, and that EOPs mediate a negative feedback regulation on LH secretion during this period when progesterone concentrations are high.

2.2.4 Luteinizing Hormone - Lactation

Post-partum secretion of LH in the pig is characterized by high frequency pulsatile secretion of variable amplitude immediately after parturition, followed by a gradual inhibition of LH secretion within approximately 72 hours of farrowing due to suckling (De Rensis, 1993; De Rensis *et al.*, 1993a and 1993b). Several studies have shown that LH secretion is low throughout established lactation, but that as lactation progresses, tonic LH secretion increases and estrogen positive feedback systems become functional (Stevenson and Britt, 1980; Stevenson *et al.*, 1981; Edwards and Foxcroft, 1983b; Cox *et al.*, 1988; De Rensis *et al.*, 1991; Sesti and Britt, 1993a and 1993b). Sesti and Britt (1993b) have shown that pituitary concentrations of LH increase from day 14 to 28 of lactation. Furthermore, administration of the excitatory amino acid agonist, N-methyl-D,L-aspartic acid (NMDA) to lactating sows showed that readily releasable pools of GnRH, as measured by changes in LH secretion following NMDA, are not different between days 1 and 7, but then increase linearly between days 7 and 21 of lactation (Sesti and Britt, 1993a and 1994). *In vitro* GnRH release in response to potassium challenge from stalk median eminence tissue, decreased from day 14 to 28 of lactation (Sesti and Britt, 1993b), indicating that an increase in endogenous rate of GnRH release was depleting the releasable pools of the hormone.

Inhibition of LH during lactation is not due to steroidal feedback, as there are no detectable circulating steroids present during lactations as long as 37 days (Baldwin and Stabenfeldt, 1975). Parvizi *et al.* (1976) and Stevenson *et al.* (1981) ovariectomized sows during lactation to demonstrate that low plasma LH concentrations were not due to ovarian effects, but due to suckling mediated effects at the level of the hypothalamus or pituitary. Transient, partial or split weaning practices decrease suckling intensity and result in increased LH secretion, and possibly estrus, during lactation and in decreased weaning to estrus intervals (Stevenson and Britt, 1981; Thompson *et al.*, 1981; Stevenson *et al.*, 1984; Armstrong *et al.*, 1988a and 1988b). Cox and Britt (1982c) showed that there was a

significant increase in GnRH concentrations in the stalk median eminence within 60 hours of complete weaning. Studies with zero weaned sows, which have their piglets removed at birth or shortly thereafter, have shown that the high levels of LH immediately post-partum are maintained in the absence of suckling (De Rensis, 1993; De Rensis *et al.*, 1993b). Collectively, these studies suggest that suckling acts at the hypothalamic level to inhibit GnRH, and thus LH secretion.

Several similar studies have provided evidence that the suckling mediated inhibition of LH secretion is via an opioidergic inhibition of GnRH release from the hypothalamus (Barb *et al.*, 1986b; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988; De Rensis *et al.*, 1993a). In all of these studies, administration of NAL increased episodic LH release and decreased PRL secretion. In contrast, NAL administered immediately post-partum to lactating sows could not prevent the suckling induced suppression in LH secretion (De Rensis *et al.*, 1993a), indicating that the opioids suppress LH secretion during established, but not in very early lactation. De Rensis (1993) administered morphine to suckled and zero-weaned sows in the immediate post-partum period and at day 10 of lactation. Morphine suppressed the persistent high concentrations of LH normally seen in zero-weaned sows and suppressed LH secretion on day 10 in suckled sows; however, there was no clear effect of morphine treatment in the immediate post-partum period in the suckled sows. These data suggest that the initial suckling induced suppression of LH secretion is not mediated by the opioids, and as yet, the mechanism involved remains to be determined.

Weaning consistently results in a significant increase in LH concentrations (Bevers *et al.*, 1983; Edwards and Foxcroft, 1983b; Shaw and Foxcroft, 1985; Foxcroft *et al.*, 1987; Sesti and Britt, 1993b). Consistent with evidence for opioidergic regulation of LH secretion during lactation, Armstrong *et al.* (1988b) demonstrated that an infusion of morphine prevents the increase in LH secretion typically seen with transient weaning and delays the onset of estrus after weaning.

2.2.5 Follicle Stimulating Hormone

Follicle stimulating hormone is also released from the anterior pituitary in response to GnRH stimulation (Schally *et al.*, 1971). However, unlike LH which requires continual GnRH stimulation for release, FSH is only partially dependent on continuous GnRH stimulation (Culler and Negro-Vilar, 1986; Sanchez *et al.*, 1994). Two separate studies in swine (Estienne *et al.*, 1990a; Barb *et al.*, 1992a) have shown that intraventricular morphine administration suppresses FSH secretion, providing evidence for participation of GnRH in the regulation of both LH and FSH in this species. In contrast to LH, FSH is also regulated by the ovarian peptides, inhibin and activin, as well as the gonadal steroids (Lumpkin *et al.*, 1984; Britt *et al.*, 1985; Culler and Negro-Vilar, 1986; Vale *et al.*, 1986; Roser *et al.*, 1994). GnRH differentially regulates LH and FSH secretion and mRNA levels of peptide subunits (α, ßLH and ßFSH). High frequency GnRH pulses favour the production of LH, as in the late follicular phase, and slower frequencies favour FSH production, as in the luteal and early follicular phase (Dalkin *et al.*, 1989 (rat); Haisenleder *et al.*, 1991(rat); Jayes *et al.*, 1997 (pig); Vizcarra *et al.*, 1997 (cow)). GnRH pulse amplitude has also been shown to have an

effect on subunit mRNA levels; lower amplitude pulses are more conducive to the production of ß subunits, whereas higher amplitude pulses stimulate production of α subunits (Haisenleder *et al.*, 1990). These studies provide evidence for differential regulation of LH and FSH from the anterior pituitary by the same hypothalamic releasing factor, and suggest methods, such as altering GnRH pulse amplitude and frequency, by which reproduction can be controlled.

In the sow, as in other female mammals, FSH recruits and stimulates the growth of small to medium sized ovarian follicles in the cycling female, but must work in conjunction with LH to cause estrogen secretion from large preovulatory follicles, (Stevenson *et al.*, 1981; as reviewed by Chappel and Howles, 1991). Normally, sows exhibit an endogenous FSH surge at the same time as the LH surge, although it is generally of lower relative magnitude than the LH surge. However, sows which had been weaned at 3 weeks compared with 5 weeks of lactation, failed to exhibit an FSH surge (Edwards and Foxcroft, 1983a and 1983b). The FSH surge is due to the massive release of GnRH at the time of the LH surge, and it is felt that the FSH surge has no real physiological importance to ovulation (Edwards and Foxcroft, 1983b).

Cox and Britt (1982a) found high concentrations of FSH in the pituitary throughout lactation in the sow, suggesting that FSH continues to be synthesized even though GnRH release is largely suppressed. To offset this lack of a GnRH drive, inhibition from inhibin is minimized, and FSH concentrations gradually increase throughout lactation (Stevenson *et al.*, 1981; Duggan *et al.*, 1982; Edwards and Foxcroft, 1983b). As the intensity of the suckling stimulus decreases during lactation, LH episodic secretion also increases and at the ovarian level this leads to a gradual increase in the development of medium and large sized follicles (Kunavongkrit et al, 1982). Sesti and Britt (1993b) found that pituitary and circulating FSH concentrations were greater in sows at day 28 versus day 14 of lactation and, as with LH, there was a linear increase in FSH released in response to NMDA stimulation between days 1 and 21 of lactation (Sesti and Britt, 1993a). De Rensis *et al.* (1993a) did not report an increase in FSH in response to NAL treatment in lactating sows, suggesting that regulation of FSH depends more on ovarian factors, or the lack thereof, than hypothalamic factors. Shaw and Foxcroft (1985) found that there is an increase in FSH concentrations between 12 hours before and the 12 hours immediately following weaning, but this increase did not reach significance until 36 and 48 hours after weaning. However, there was no correlation between FSH levels and weaning to estrus interval (Shaw and Foxcroft, 1985). Conversely, in a later study, there was no consistent FSH response to weaning, and in fact FSH concentrations decreased from 12 to 36 hours after weaning (Foxcroft *et al.*, 1987).

These studies indicate that during lactation, even though the GnRH pulse generator has slowed due to suckling mediated inhibition, FSH continues to be secreted due to the absence of the gonadal feedback mechanisms. Indeed, the slow frequency of GnRH pulses, typical of lactation, favour the synthesis of FSH. This mechanism ensures that a new crop of small to medium sized follicles are available for recruitment, so that shortly after weaning, final maturation and ovulation will occur.

2.2.6 Prolactin - Estrous Cycle

Although, prolactin (PRL) is a hormone with varied activities and is released under several different physiological paradigms, in the pig it is mainly lactogenic and, later in gestation, luteotropic in its actions (Taverne *et al.*, 1982; Gregoraszczuk, 1990; Szafranska *et al.*, 1992; Szafranska and Tilton, 1993). PRL producing lactotropes have an intrinsic ability to secrete PRL without stimulation and therefore must be under chronic inhibition to regulate secretion. Dopamine (DA) is considered to be the major putative PRL inhibiting factor in the hypothalamo-pituitary system of mammals (as reviewed by Ben-Jonathan, 1985). The tuberoinfundibular hypothalamic DA neurons are responsible for PRL inhibition via a D_2 receptor on the lactotropes. These DA neurons release dopamine into the portal vessels and may also reach the lactotropes via the short portal vessels of the posterior pituitary (as reviewed by Neill, 1988). In pigs, DA also inhibits PRL secretion, as both administration of haloperidol (HAL), a dopamine antagonist, or hypophysial stalk transection, increased PRL secretion in cyclic gilts (Kesner *et al.*, 1989a; Anderson *et al.*, 1991; Kraeling *et al.*, 1994). HAL failed to increase PRL secretion in HST gilts, likely because PRL secretion was already maximal (Anderson *et al.*, 1991). Conversely, treatment of HST, intact and OVX gilts with the DA agonist, bromocriptine (CB-154), suppressed PRL secretion (Kraeling *et al.*, 1982 and 1994).

Certainly in the rat, PRL appears to have several potential releasing hormones, among them serotonin, angiotensin, oxytocin, EOP, vasoactive intestinal polypeptide and thyrotropin releasing hormone (TRH) (as reviewed by Weiner *et al.*, 1988). Anderson *et al.* (1991) and Kraeling *et al.* (1994) have recently demonstrated that in the pig, TRH stimulates PRL secretion from the pituitary of HST gilts. The EOP have also been shown to potently stimulate PRL secretion under various physiological conditions, such as the responses to stress and suckling (Barb *et al.*, 1986b; Rushen *et al.*, 1993). Intraventricular administration of morphine to mature and prepubertal OVX gilts stimulated PRL secretion (Estienne *et al.*, 1990a; Barb *et al.*, 1992a). Conversely, during the estrous cycle, Barb *et al.* (1985, 1986a, and 1992a) reported that opioid antagonism with NAL increased PRL secretion in luteal phase and OVX progesterone treated gilts, but not in follicular phase or OVX gilts. In a later study, Chang *et al.* (1993a) showed that NAL increased PRL secretion in progesterone treated OVX gilts, and that opioidergic regulation of PRL secretion was independent of the noradrenergic system. The conflicting data derived from morphine and NAL administration in these experiments may be explained by the presence of opioidergic autoreceptors on ß-endorphin neurons in the rat arcuate nucleus (Zhang *et al.*, 1996). The antagonism of these receptors with NAL would actually result in an increase in opioidergic tone and therefore, stimulation of PRL secretion.

Administration of NMDA has been shown to stimulate PRL secretion in OVX and steroid treated OVX gilts (Barb *et al.* 1992b; Chang *et al.*, 1993b). Following NAL administration this effect is abolished, indicating a role for excitatory amino acids and EOP in PRL secretion (Chang *et al.*, 1993b). This stimulatory action on PRL is thought to be mediated mainly by the EOP inhibition of DA. They may also act via stimulation of another PRL secretagogue, as yet unidentified in the pig.

Unlike the rat, in the pig there is no daily surge of PRL during the estrous cycle. However there are two distinct PRL surges during the estrous cycle, one occurring during the early follicular phase and the other during estrus (Van Landeghem and Van de Wiel, 1977; Dusza and Krzymowska, 1979). The concomitant LH and PRL surges at estrus are due to the actions of estrogen (Stevenson *et al.*, 1981). It has been suggested that PRL functions in an inhibitory manner during growth and development of ovarian follicles and becomes stimulatory when the follicles are mature. However, treatment of sows with CB-154, while abolishing the two PRL surges during the cycle, did not affect LH surge secretion or the initiation of the subsequent estrous cycle (Dusza *et al.*, 1983).

2.2.7 Prolactin - Gestation

Prolactin secretion throughout gestation is pulsatile in nature and does not vary appreciably for the first 90 days; however, it then increases on or before day 110 of gestation (Benjaminsen, 1981; *et al.*, 1978/1979; Kraeling *et al.*, 1992a). Dusza and Krzymowska (1981) report that the increase in PRL secretion occurs approximately two days prior to, or at, parturition. This PRL increase is thought to initiate lactogenesis, as treatment with CB-154 at this time prevents the late gestational surge of PRL and subsequent lactation is impaired (Taverne *et al.*, 1982).

Although the data are somewhat equivocal, PRL has been suggested as a possible luteotropic factor during gestation in the pig. As discussed earlier, LH is considered to be luteotropic during pregnancy in the sow (Ziecik *et al.*, 1982/1983; Wiesak, 1985; Szafranska *et al.*, 1992), but there are several studies which also suggest a role for PRL in maintenance of the CL. Rolland *et al.* (1976) showed that during the first half of gestation the number of PRL binding sites on the CL increase, reaching a maximum number by day 60 (Jammes *et al.*, 1985). *In vitro*, large luteal cells secrete progesterone in response to PRL (Gregoraszczuk, 1990) and PRL stimulated progesterone secretion from luteal tissue taken from gilts on day 80 of gestation, in which LH had previously been immunoneutralized (Szafranska *et al.*, 1992).

Treatment with CB-154 reduced PRL concentrations and resulted in early luteolysis and early parturition (Taverne *et al.*, 1982). These results are in direct contrast to Szafranska and Ziecik (1990), who report no effect of CB-154 on luteal function in the pregnant gilt, even though PRL and LH were suppressed by treatment. Short-term inhibition of PRL secretion at days 40 and 70 of gestation with NAL did not affect the outcome of pregnancy, although it does suggest a role for EOP regulation of PRL secretion during gestation in the sow (Szafranska and Tilton, 1995). Furthermore, on days 60-66 of gestation gilts were made hyperprolactinemic using HAL (Szafranska and Tilton, 1993). This treatment, while resulting in reduced LH secretion, increased progesterone concentrations and no abortions occurred. These experiments strongly suggest a role for PRL as a luteotropic factor in the later half of pregnancy.

2.2.8 Prolactin - Lactation

PRL secretion is responsible for the maintenance of lactation and the regulation of gene expression in mammary tissue, thus stimulating the production of many milk components (as reviewed by Rillema *et al.*, 1988). Several studies measuring PRL concentrations throughout lactation in the sow have shown that the concentrations are higher at the outset of lactation, when the demand for milk is high, than they are as lactation progresses; however, these concentrations are still much higher than basal PRL concentrations seen during the estrous cycle (Bevers *et al.*, 1978; Van Landeghem and Van de Wiel, 1978; Edwards and Foxcroft, 1983b; Dusza *et al.*, 1987). PRL concentrations in suckled sows were high immediately after farrowing compared with low PRL concentrations in zero weaned sows at the same time (De Rensis, 1993; De Rensis *et al.*, 1993b). PRL secretion decreased in response to transient weaning and increased again when the piglets were replaced (Armstrong *et al.*, 1988a and 1988b). After weaning there is a dramatic decrease in circulating PRL concentrations (Benjaminsen, 1981; Edwards and Foxcroft, 1983b; Shaw and Foxcroft, 1985; Foxcroft *et al.*, 1987; Rojkittikhun *et al.*, 1991b).

During lactation, Van de Wiel *et al.* (1981) noted that during periods of decreased PRL secretion there was a marked increase in LH secretion. Booman *et al.* (1983) demonstrated that this relationship between PRL and LH secretion exists after weaning. Foxcroft *et al.* (1987) found that there was a drastic decrease in PRL secretion shortly after weaning, and that PRL remained low while LH secretion gradually increased. Armstrong *et al.* (1988a and 1988b) noted that during transient weaning during lactation, LH secretion increased and PRL secretion decreased. These data show a distinct inverse relationship between PRL and LH secretion in the lactating and weaned sow. The inverse relationship between LH and PRL secretion during lactation and at weaning resulted in the theory that high PRL concentrations during lactation mediated lactational anestrus. Experiments in the sow which attempt to clarify this concept are discussed in the next section.

Several studies have since demonstrated that the EOP are responsible for mediating the inverse relationship between LH and PRL secretion in the sow during lactation and at weaning. In the studies by Mattioli *et al.* (1986), Armstrong *et al.* (1988a), and De Rensis *et al.* (1993a), LH secretion increased, and PRL secretion decreased in response to treatment with NAL during established lactation. These authors report that suckling bouts still occurred during treatment with NAL, therefore, it appears that endogenous opioids released in response to suckling mediate the effects on these two hormones in lactation. Interestingly, De Rensis *et al.* (1993a) report that like LH, PRL secretion is not affected by administration of NAL in the immediate post-partum period. In fact, there is no response until at least 78 hours post-partum, which may indicate that opioidergic receptors in the hypothalamus downregulate during parturition, and that upregulation requires 78 hours. Morphine administration to suckled and transiently weaned sows inhibited PRL secretion (Armstrong *et al.*, 1988b). Similarly, morphine failed to stimulate PRL secretion in the immediate post-partum period in both suckled and zero-weaned sows, and in fact actually inhibited PRL secretion on day 10 of lactation (De Rensis, 1993). These authors suggest that lactating sows are refractory to morphine stimulus due to high circulating endogenous opioids brought about

by suckling.

2.2.9 Prolactin and LH Interactions in the Sow - Is Hyperprolactinemia the Cause of Lactational Anestrus?

Hyperprolactinemia is frequently associated with clinical conditions in which the normal mechanisms for inhibition by DA on PRL appear to be dysfunctional, such as PRL secreting tumours and neuroendocrine dysfunction (psychological or diseases such as Parkinsons and Tourrettes). Hyperprolactinemia can also be experimentally induced by transplanting pituitaries under the kidney capsule, thereby removing the pituitary from DA inhibition from the hypothalamus, or by pharmacologically blocking dopaminergic inhibition of PRL using a DA antagonist such as haloperidol, domperidone, or pimozide (as reviewed by Benker *et al.*, 1990). Hyperprolactinemia also occurs in a lesser form during lactation, and is characterized in women by higher than normal prolactin secretion in response to suckling episodes, and reduced LH concentrations and a delayed resumption of ovarian activity (Kremer *et al.*, 1989; Tay *et al.*, 1993).

As discussed earlier, in the sow during lactation, it is known that opioid peptides mediate the release of PRL while simultaneously inhibiting the release of GnRH and LH, resulting in an inverse relationship between PRL and LH secretion. These observations prompted many investigations into the role that PRL plays in the suppression of GnRH/LH and lactational anestrus. Bromocriptine treatment suppressed both PRL and LH secretion in sows between day 28 and 30 of lactation (Kraeling *et al.*, 1982). However, in a study by Bevers *et al.* (1983), although CB-154 from day 14 to day 22 of lactation reduced PRL levels in lactating sows to those seen in weaned animals, this same treatment significantly increased LH levels. Furthermore, Mattioli and Seren (1985) hypothesized that CB-154 treatment would mimic the endocrine changes associated with weaning, ie, that suppressed PRL would permit increased LH secretion and a resumption of ovarian activity. They assumed that as return to estrus occurs approximately 5 days after weaning, CB-154 treatment 5 days prior to weaning would cause estrus immediately after weaning. In contrast to the earlier 2 studies discussed, while CB-154 administered from day 15 to 20 of lactation decreased PRL secretion, treatment failed to affect LH secretion and sows still returned to estrus at approximately 5 days following weaning.

Furthermore, in a study that compared sows which returned to estrus within 10 days of weaning and sows which had a delayed return to estrus, Benjaminsen (1981) found no differences in plasma PRL levels between the two groups. Edwards and Foxcroft (1983b) also found no differences in post-weaning PRL concentrations between sows which failed to return to estrus or ovulate and those which did. To further study the role of PRL in lactational infertility, Dusza *et al.* (1984) administered exogenous PRL after weaning until the onset of estrus and found it had no effect on plasma LH concentrations and that the preovulatory LH surge occurred in all animals, irrespective of treatment with PRL. Conversely, Booman *et al.* (1982) infused sows with PRL for 24h post-weaning and found that mean and basal plasma LH concentrations and LH pulse frequency were significantly reduced compared with saline infused controls. Both the studies during lactation and after

weaning yielded varying results, under some experimental conditions it was shown that suppression of PRL could stimulate LH secretion, while maintaining PRL concentrations at artificially high levels post weaning also had mixed results. It is therefore difficult to conclude that high PRL concentrations alone mediate lactational anestrus in the sow.

Yet, one other possibility remains, and that is that the PRL inhibiting factor, dopamine, is responsible for mediating the effects of lactational anestrus. This may be true for the immediate post-partum period when there doesn't appear to be an opioid mediated mechanism in place for the suckling induced suppression of LH secretion. At this time, PRL levels are high (Bevers *et al.*, 1978; Van Landeghem and Van de Wiel, 1978; Edwards and Foxcroft, 1983b), so DA secretion from the hypothalamus would be suppressed. Studies in the rat and human have shown that in the absence of a dopaminergic influence, GnRH/LH secretion is inhibited (Rotsztejn *et al.*, 1977; Rasmussen *et al.*, 1986; Li and Pelletier, 1992). In the sow, the results of Bevers *et al.* (1983) would be consistent with this theory, in that treatment with the DA agonist, CB-154, stimulated LH secretion. Conversely, several other studies in the rat (Tasaka *et al.*, 1985), sheep (Tortonese and Lincoln, 1994) and human (Andersen *et al.*, 1987) suggest that DA is inhibitory to GnRH/LH secretion, and so the administration of CB-154 would suppress LH secretion (Kraeling *et al.*, 1982). Treatment of OVX gilts with CB-154 significantly reduced the sensitivity of the pituitary to exogenous GnRH (Mattioli and Seren, 1985). Plasma LH concentrations were lower and a response took longer in gilts treated with CB-154, compared with controls. Again, the results from these studies are inconclusive, LH secretion is stimulated or inhibited in the presence or absence of a dopaminergic tone. Further investigation is warranted in the regulation of GnRH/LH secretion in the immediate post-partum period, when opioids do not contribute to the suckling induced suppression of LH secretion.

2.3 The Hypothalamic GnRH Pulse Generator

"The GnRH pulse generator is defined as the neuronal construct that eventuates in the pulsatile discharge of LH into the peripheral circulation. The activity of the GnRH pulse generator can be assessed by the monitoring of pulsatile LH secretion and/or any antecedent or associated event such as GnRH release, the electrophysiological manifestations of associated neurosecretory processes, as used here, or other cognate phenomena." (O'Byrne *et al.*, 1993).

Certainly the most popular concept in the regulation of hypothalamic GnRH secretion is that of the "GnRH pulse generator". In the early work in E. Knobil's laboratory with OVX monkeys, LH was secreted in rhythmic bursts, approximately 1 every hour (Dierschke *et al.*, 1970). Following this discovery, there were several views as to the regulation of these pulses, some favouring the concept that LH regulated its own secretion via short and long feed back loops (as reviewed by Knobil, 1989). However, that theory was put aside, and it was determined that each pulse of LH was the consequence of a bolus of GnRH discharged into the pituitary portal vessels (Clarke and Cummins, 1982). In monkeys (Krey *et al.*, 1975) and rats (Blake and Sawyer, 1974), the pulse generator is located within the MBH, as surgical deafferentation of the hypothalamus does not disrupt pulsatile LH secretion.

Through a variety of techniques, several inputs to the GnRH pulse generator have been elucidated. One of the most frequently used techniques is the measurement of multi unit activity (MUA), whereby a recording electrode is placed within the hypothalamus which then measures the electrical activity of neural action potentials (reviewed by Mori *et al.*, 1996). Knobil (1981) showed that each MUA volley related to the initiation of an LH pulse in OVX anaesthetised rhesus monkeys. Since then, MUA correlates to LH secretion have been identified in OVX conscious rhesus monkeys (Wilson *et al.*, 1984), OVX rats (Kimura *et al.*, 1991; Nishihara *et al.*, 1991) and goats (Mori *et al.*, 1991; Tanaka *et al.*, 1995). Another method frequently used is *in vitro* perfusion of hypothalamic tissue, and the subsequent quantification of GnRH in the samples collected in response to various drugs (Nowak and Swerdloff, 1985; Clough *et al.*, 1988; Masotto *et al.*, 1989).

However, there is still ongoing debate as to what the inputs to the pulse generator are. Kesner *et al.* (1986) abolished MUA volleys, in the MBH of OVX rhesus monkeys, following the administration of morphine, and reinitiated them following the administration of NAL. Administration of the non-specific α-noradrenergic receptor antagonist, phentolamine, the α_1-antagonists, phenoxybenzamine and prazosin, and the antidopaminergic drug, metoclopramide, arrested MUA volleys and the associated LH pulses in OVX monkeys (Kaufman *et al.*, 1985). The $GABA_A$ receptor agonist, muscimol, increased the interval between MUA volleys in OVX rats (Kimura *et al.*, 1993). Furthermore, GABA, administered directly into the PO/AH of OVX rats via push pull perfusion cannulation completely abolished LH pulsatility (Jarry *et al.*, 1991). These studies suggest that the opioidergic, noradrenergic, dopaminergic and GABAergic systems are all possible neural inputs to the GnRH pulse generator.

2.4 The GnRH Neuron's Intrinsic Ability to Pulse

Another possibility is that GnRH neurons have an intrinsic pulsatility and, therefore, they in themselves may be the pulse generator. A single injection of clonidine, an α-adrenergic receptor agonist, was able to restore sustained pulsatile LH secretion in OVX rats which had previously been treated with the noradrenergic synthesis inhibitor, diethyldithiocarbamate (DDC) (Estes *et al.*, 1982). This raised the possibility that the central noradrenergic system may only be permissive to GnRH/LH secretion, but that the ability to pulse resides within the GnRH system. Furthermore, surgical deafferentation of the noradrenergic tract in OVX rats results in a temporary cessation of pulsatile LH secretion. However, pulsatility was restored within 4 weeks after surgery. Phenoxybenzamine, a noradrenergic antagonist did not affect LH pulsatility in these animals, indicating that an alternative mechanism was generating GnRH pulses (Clifton and Steiner, 1985). Similarly, i.c.v. injection of a neurotoxin, which selectively depletes norepinephrine (NE), resulted in a complete depletion of hypothalamic NE within 52 hours of administration. Despite the loss of a stimulatory noradrenergic drive, LH secretion in these rats was not different from untreated controls (Leonhardt *et al.*, 1991). Clifton and Steiner (1985) make an interesting analogy, that GnRH neurons are similar to cardiac cells, they may exhibit spontaneous pulsatile activity, but just as the heart does not require a stimulatory input from NE to beat,

GnRH neurons do not require a noradrenergic input to pulse.

Intrinsic pulsatility has been shown to be an inherent characteristic of a line of immortalized GnRH neurons (Krsmanovic *et al.*, 1992; Martinez De La Escalera *et al.*, 1992; Wetzel *et al.*, 1992). GT1-1 cells exhibit an oscillating pattern of GnRH release when cultured on cover slips, in the absence of any other neural inputs (Krsmanovic *et al.*, 1992; Martinez De La Escalera *et al.*, 1992). Pulsatility was inhibited in a Ca^{2+} deficient medium, and by Ca^{2+} channel antagonists. GnRH secretory activity was similar to that observed *in vivo*, suggesting that the pulse generating mechanism of the GT1-1 cells may be voltage sensitive Ca^{2+} channels (Krsmanovic *et al.*, 1992). GnRH secretion from GT1-1 cells cultured in a perfusion chamber, on two opposing cover slips, became synchronized; perhaps indicating the presence of a "diffusible mediator" (Martinez De La Escalera *et al.*, 1992). The authors suggest that a logical choice for the mediator would be GnRH secreted from the cells.

Regardless of the viewpoint taken, "pulse generator" or "intrinsic ability", it is clear that many interacting neuronal systems modulate the pattern of GnRH secretion, thereby affecting the frequency and amplitude of LH pulses. These are discussed in the following sections.

2.5.1 Hypothalamic Regulation of GnRH Secretion

The involvement of the many hypothalamic factors thought to be involved in the central regulation of GnRH, and thus LH, secretion is largely neuromodulatory, and varies dependent on the physiological status and steroidal environment of the animal. Key factors regulating GnRH secretion include GnRH itself, norepinephrine (NE), endogenous opioid peptides (EOP), excitatory amino acids (EAA) and γ-aminobutyric acid (GABA). Others which may play a minor role in GnRH secretion are dopamine (DA), neuropeptide Y (NPY), and serotonin (5-HT). As discussed earlier, it is well established that pulsatile LH secretion relies upon signals from the hypothalamic GnRH pulse generator. However, there has been a lot of controversy surrounding the factors that drive the pulse generator and the many complex interactions between neural inputs which regulate or modulate GnRH secretion.

2.5.2 Gonadotropin Releasing Hormone

Results from several studies have shown that GnRH regulates its own pulsatile secretion. GnRH acting centrally, in an inhibitory autofeedback loop, can inhibit GnRH secretion, and therefore, LH release from the pituitary. Conversely, the synchronization of pulsatile GnRH secretion must involve communication between, or stimulation of, adjacent GnRH neurons.

In the OVX ewe, i.c.v. administration of GnRH inhibited pulse generator activity, significantly decreasing mean LH concentration and pulse frequency in a dose dependent manner (Naylor *et al.*, 1989). As these results were obtained in OVX animals and steroids exert important modulatory effects on the pulse generator, it would be important to verify these results in the presence of estrogen or progesterone using physiological doses of GnRH. Bourguignon *et al.* (1990) showed that the administration of a GnRH superagonist to

hypothalamic explants of male rats resulted in a 2-fold increase in the GnRH interpulse interval. An additional *in vitro* study demonstrates that the breakdown product of GnRH, 1-5 GnRH, also inhibits pulsatile GnRH secretion from hypothalamic explants obtained from male rats (Bourguignon *et al.*, 1994). In OVX and luteal phase ewes, peripheral administration of the GnRH antagonist, Nal-Glu, increased LH pulse frequency, amplitude, and total and basal LH concentrations (Padmanabhan *et al.*, 1995). These authors suggest that Nal-Glu activated either a shortloop feedback system, involving the LH regulation of GnRH release, or an ultra-shortloop feedback system, whereby GnRH itself regulates GnRH release.

GnRH may also be responsible for the stimulation of its own secretion. A synchronous firing of all GnRH neurons must occur in order for a pulse of GnRH to be delivered into the portal vessels (Wilson *et al.*, 1984). In order for this to occur, some form of crosstalk between GnRH neurons must occur. Anatomically this is possible, as GnRH synapses have been located on the dendrites and perikarya of other GnRH neurons in the MPOA of rats (Leranth *et al.*, 1985a; Witkin *et al.*, 1987; Witkin *et al.*, 1995), monkeys (Witkin *et al.*, 1995), pigs (Kineman *et al.*, 1988), cattle (Leshin *et al.*, 1988) and sheep (Lehman *et al.*, 1986). In the rat, approximately fifty percent of all identified GnRH neurons project to the portal vessels in the ME, while the remainder make synaptic contacts with other neurons (Pelletier, 1987). Electron microscopic studies identified paired and grouped GnRH neurons in the hypothalamus of rats and monkeys (Witkin *et al.*, 1995). Many of these paired neurons had formed cytoplasmic bridges or regions of confluence between the cells, which were not found between GnRH neurons and other unidentified neurons. These cytoplasmic connections could be the linkage required for synchronization of the GnRH system.

Hiruma *et al.* (1989) found that injecting GnRH into the MPOA of OVX, estrogen primed rats and intact proestrous rats stimulated GnRH secretion, and an LH surge in the proestrous animals. Administration of the long-acting GnRH antagonist, antide, into the MPOA of diestrous rats disrupted the estrous cycle for as few as 11 days and as many as 100 days in some animals, indicating that functional GnRH receptors within the hypothalamus are a prerequisite for normal cyclicity in the rat (Weesner and Pfaff, 1994).

2.5.3 Endogenous Opioid Peptides

There are three categories of endogenous opioid peptides (EOP), the endorphins, enkephalins (met- and leu-) and dynorphins, and each is processed from separate precursor molecules, proopiomelanocortin (POMC), pre-proenkephalin and pre-prodynorphin, respectively (as reviewed by Brooks *et al.*, 1986). Three major types of opioid receptors have also been identified within the hypothalamus and pituitary, mu (μ), kappa (k) and delta (δ), each with distinct pharmacological properties, although these do tend to overlap (for review see Paterson *et al.*, 1983). POMC derived ß-endorphin, and perhaps the enkephalins, are considered to be the endogenous ligand for μ-receptors, enkephalins are thought to be endogenous ligands for δ-receptors and dynorphins are the ligands for k-receptors (reviewed by Corbett *et al.*, 1993). Recently, a novel post-synaptic μδ-receptor complex has been suggested which is pharmacologically different than the pre-synaptic μ- and δ-receptors (Schoffelmeer *et al.*, 1992). However, μ-opioid receptors appear to be the most likely

receptor type regulating GnRH secretion from the hypothalamus (Pfeiffer *et al.*, 1983; Walsh and Clarke, 1996).

Immunocytochemistry has identified that POMC or ß-endorphin neurons originating in the ARC nucleus make synaptic contacts with GnRH neurons in the MPOA of the monkey (Thind and Goldsmith, 1988), rat (Leranth *et al.*, 1988a; Chen *et al.*, 1989a), sheep (Conover *et al.*, 1993), cattle (Leshin *et al.*, 1988 and 1992b) and pig (Kineman *et al.*, 1989). Both Arvidsson *et al.* (1995) and Ding *et al.* (1996) have identified the μ-opioid receptor, MOR1, in the MPOA and anterior hypothalamus of rats, where they are located mainly post-synaptically, and to a much lesser extent, pre-synaptically. When these receptors are activated, they may function either to reduce the receptivity of the post-synaptic neuron to incoming stimuli, or inhibit neurotransmitter release from presynaptic neurons (Ding *et al.*, 1996). Both of these have been suggested as mechanisms whereby the EOP could inhibit GnRH secretion.

The main body of evidence is in agreement, that the EOP are inhibitory to LH secretion and that this inhibition occurs at a hypothalamic level, via inhibition of GnRH secretion (Blank and Roberts, 1982). In an early study, Pang *et al.* (1977) discovered that morphine inhibited the LH surge in proestrous rats and blocked ovulation, and that this effect was reversed when the opioid antagonist, NAL, was given. This effect was mediated at the hypothalamic level as exogenous GnRH was able to release LH from the pituitary in the morphine blocked rats. Morphine disrupts the MUA volleys, associated with LH pulsatility, in the hypothalamus of OVX rhesus monkeys (Kesner *et al.*, 1986). This effect was reversible by NAL, but NAL alone had no effect on MUA volleys.

The stage of the estrous cycle, and therefore the steroidal environment, has important effects on whether the EOP affect LH secretion. There is some evidence in the rat (Jirikowski *et al.*, 1986) and the sheep (Lehman and Karsch, 1993) that ß-endorphin neurons which originate within the MBH and project to the MPOA contain estrogen receptors. A subpopulation of ARC nucleus ß-endorphin neurons in OVX, steroid primed, rhesus monkeys contain progesterone receptors (Bethea and Widmann, 1996). Since GnRH neurons do not express steroid receptors (Herbison *et al.*, 1992, 1993 and 1995), opioidergic neurons expressing steroid receptors may mediate the negative feedback effects of gonadal steroids on GnRH secretion.

Bhanot and Wilkinson (1984), have shown that the inhibitory effects of opioids are steroid dependent, as NAL failed to increase, and FK 33-824, a synthetic met-enkephalin, did not inhibit LH secretion in long-term OVX rats. The effects of both the antagonist and agonist were restored when the rats were primed with estradiol. Gabriel *et al.* (1983) demonstrated that NAL was effective in disinhibiting LH secretion from opioidergic inhibition at all stages of the estrous cycle and in estradiol primed OVX rats. Conflicting results from studies in cattle have the EOP regulating LH secretion in the follicular (Short *et al.*, 1987) and the luteal phase (Mahmoud *et al.*, 1989; Stumpf *et al.*, 1993) of the cycle; irrespective of the stage of the cycle, the presence of steroids appear to be necessary for the EOP to have any effect on LH secretion in cattle. Administration of estradiol to long-term OVX monkeys significantly increased the interval between MUA volleys (Grosser *et al.*, 1993). As these effects were similar to the effects of morphine administration on MUA volleys (Kesner *et al.*,

1986), Grosser *et al.* (1993) administered NAL to estrogen primed OVX monkeys, which subsequently decreased the interval between MUA volleys. Naloxone was only effective in releasing LH in OVX pony mares when previously treated with estradiol, alone, or combined with progesterone; treatment in OVX, or OVX progesterone primed, mares was ineffective (Aurich *et al.*, 1995). Intrapreoptic application of ß-endorphin and NAL, inhibited and stimulated LH secretion, respectively, in the OVX rat (Jarry *et al.*, 1995). ß-endorphin had no effect in estrogen primed rats, although NAL increased LH secretion (Jarry *et al.*, 1995), which may indicate that during estrogen negative feedback, LH secretion is already maximally suppressed and any further increases in opioid tone would have no effect.

Many studies have shown that NAL is effective in eliciting an increase in LH only during the luteal phase or in the presence of exogenous progesterone. Unlike the OVX mare, in the cyclic mare NAL administration during the follicular phase had no effect on LH secretion, and was only effective during the luteal phase (Behrens *et al.*, 1993). During the ewe's breeding season, the EOP tonically inhibit LH secretion, as NAL effectively increased LH secretion, increasing both basal secretion and pulse amplitude, during the luteal phase (Currie and Rawlings, 1987; Havern *et al.*, 1991). LH pulse amplitude was also increased by NAL in the very early follicular phase. This was likely due to residual circulating progesterone, or may indicate that pulse amplitude and frequency are regulated independently (Currie and Rawlings, 1987). Yang *et al.* (1988) demonstrated that WIN-3, an opioid antagonist, increased LH secretion in luteal phase ewes, and OVX animals in the non-breeding or the breeding season provided the animals were treated with progesterone. Intrahypothalamic immunoneutralization of ß-endorphin effectively disinhibited GnRH/LH secretion in luteal phase ewes when administered into the POA/AH (Weesner and Malven, 1990). In two similar studies by Shen *et al.* (1995) and Thom *et al.* (1996), it was found that the steroidal environment influenced the type of opioid receptors in the hypothalamus of the OVX ewe; μ-receptors were regulated by progesterone and δ-receptors by estrogen. In cyclic rhesus monkeys, the opioid antagonist, nalmefene, significantly enhanced GnRH release from the hypothalamus and reinstated a regular pattern of pulsatile LH secretion during the luteal phase, but not during the follicular phase (Pau *et al.*, 1996). Together, all of these studies are highly suggestive of a role for the endogenous opioid systems in mediating the effects of steroid negative feedback, particularly the effects of progesterone negative feedback in domestic species and primates.

Although there is an overwhelming availability of evidence that the EOP are inhibitory to GnRH/LH secretion, there is no clear evidence indicating the site of action of the EOP on the GnRH system. There are some reports that opioids inhibit the release of LH, or modify the response to GnRH stimulation directly, at the pituitary level in the rat (Cacicedo and Sánchez-Franco, 1985; Blank *et al.*, 1986; Dragatsis *et al.*, 1995), cattle (Chao *et al.*, 1986), sheep (Matteri and Moberg, 1985) and pig (Barb *et al.*, 1990). Others report that the EOP regulate GnRH secretion at the level of the GnRH neuron. GnRH neurons in OVX guinea pigs express functional μ-receptors, as the μ-opioid receptor agonist, DAMGO, hyperpolarized these cells *in vitro*, this effect was reversed by NAL (Lagrange *et al.*, 1995). In GT1-7 GnRH cells, Nazian *et al.* (1994) reported that morphine inhibited the stimulated GnRH response to isoproterenol and dopamine. Similarly, prostaglandin or estradiol

stimulated GnRH release was inhibited by the δ-receptor agonist, [D-Pen^2,D-Pen^5]enkephalin in GT1-1 GnRH cells; the agonist had no effect on unstimulated release (Maggi *et al.*, 1995). These results suggest that GnRH neurons and transformed GnRH neural cell lines express functional opioid receptors, which, when activated, appear to decrease the responsiveness of the GnRH neuron to stimulatory inputs. There is one more level at which the EOP may function to regulate GnRH secretion, and that is a presynaptic inhibition of the stimulatory noradrenergic input. This is discussed in detail in a later section.

2.5.4 Opioidergic Regulation of GnRH/LH Secretion in the Pig

As in the studies with other species, endogenous opioids are inhibitory to GnRH/LH secretion in the pig, and function only in the presence of steroids, particularly progesterone. In the gilt, NAL was able to significantly increase serum LH concentrations in the luteal phase, but not the early or late follicular phase, of the estrous cycle or in OVX gilts (Barb *et al.*, 1985 and 1986a; Okrasa *et al.*, 1990). Naloxone failed to affect LH secretion in prepubertal, mature OVX and prepubertal OVX-progesterone treated gilts, but did increase LH secretion in mature OVX-progesterone-treated gilts (Barb *et al.*, 1988). The results from this experiment suggest that although progesterone is a prerequisite for opioid inhibition of LH secretion, the systems regulating LH secretion must also undergo a maturational process. During gestation, another physiological state in which progesterone is dominant, the EOP also function to inhibit LH secretion. Naloxone stimulated LH secretion in gestation in the sow, indicating that the EOP inhibit LH secretion at day 40 (Szafranska *et al.*, 1994). Together, these studies suggest that the EOP mediate the effects of progesterone negative feedback on LH secretion during the luteal phase of the estrous cycle and during gestation.

Exogenous opioids also inhibit GnRH/LH secretion in other than progesterone dominated environments. I.c.v. administration of morphine to OVX gilts resulted in a significant decrease in serum LH concentrations and delayed the estradiol benzoate induced LH surge by more than 10 hours (Barb *et al.*, 1989; Estienne *et al.*, 1990; Kraeling *et al.*, 1992b). Infusion of the met-enkephalin analogue, FK 33-824, on day 19 of the estrous cycle (mid follicular phase) also inhibited LH secretion and NAL reversed this inhibition (Okrasa and Tilton, 1992).

During lactation, the EOP again inhibit LH secretion and stimulate the release of PRL, even though the concentrations of steroids are extremely low at this time. The stimulation of PRL is indirect via inhibition of dopamine, the major putative PRL inhibiting factor (Johnson *et al.*, 1991; Soaje and Deis, 1994). As already discussed, in the sow, there is an inverse relationship between LH and PRL secretion during lactation and after weaning (Bevers *et al.*, 1983; Shaw and Foxcroft, 1985; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a and 1988b; De Rensis *et al.*, 1993a). Due to the inverse relationship, it was believed that it was PRL that was perhaps responsible for the suppression of LH secretion during lactation. In a series of similar studies, Barb *et al.* (1986b), Mattioli *et al.* (1986), and Armstrong *et al.* (1988a) found that NAL increased episodic LH release but decreased peripheral PRL during established lactation in the sow; following weaning, NAL had no effect on LH secretion. Administration of morphine to transiently weaned sows and weaned sows, prevented the

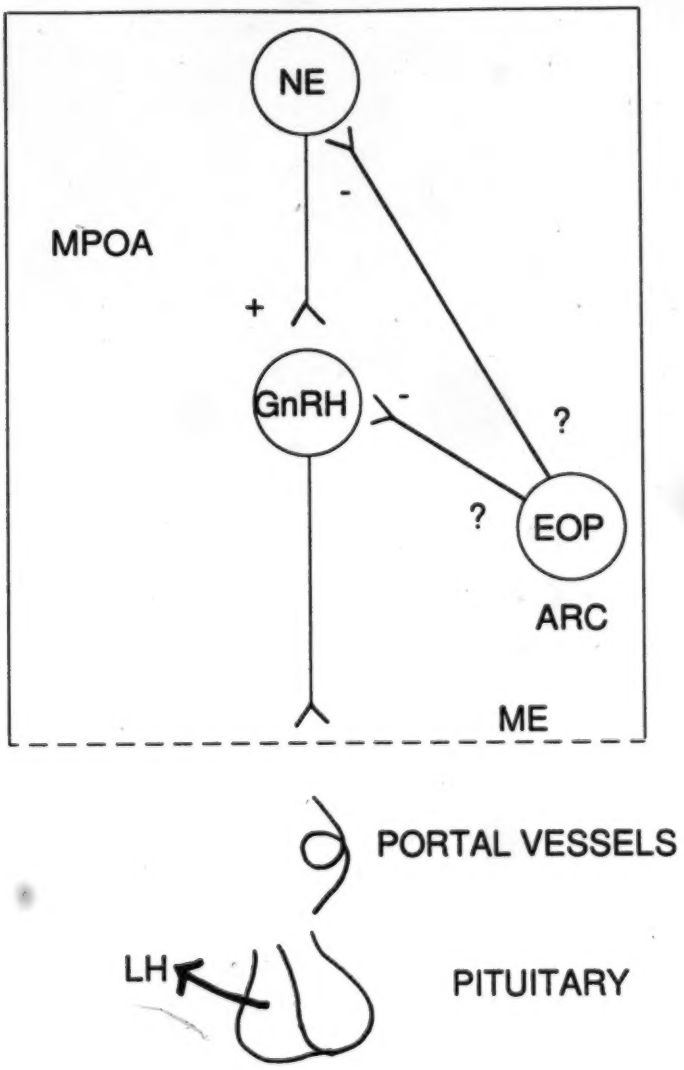
increase in LH secretion normally associated with the removal of the suckling stimulus and prolonged the weaning to estrus interval, respectively (Armstrong *et al.*, 1988b). De Rensis *et al.* (1993a) also showed that NAL administered on day 10 of lactation (the positive control for these experiments) increased LH and decreased PRL secretion, but was unable to elicit a response prior to 78 hours post-partum. Available evidence supports a role for endogenous opioids in the suckling induced inhibition of LH secretion during mid- and late lactation, but not early in lactation.

It has been suggested that the EOP function at the level of the hypothalamus to decrease GnRH release and inhibit LH secretion (reviewed by Kraeling and Barb, 1990). Anatomical studies have shown that GnRH perikarya and POMC immunoreactive neurons are located in the MPOA of the porcine hypothalamus and that further interactions may occur between GnRH axons and POMC neurons in the MBH (Kineman *et al.*, 1988 and 1989). Kumar *et al.* (1991) measured high concentrations of met-enkephalin in the MPOA and MBH of luteal phase gilts. In the study by Estienne *et al.* (1990a) i.c.v. morphine administration suppressed serum LH levels but did not decrease pituitary responsiveness to exogenously administered GnRH inferring that morphine was acting at the hypothalamic level. Consistent with this suggestion, in a perfusion study using MPOA tissue obtained from follicular phase gilts, NAL stimulated basal GnRH and morphine inhibited GnRH secretion *in vitro* (Barb *et al.*, 1994). In part two of the Barb *et al.* (1994) study, MPOA tissue from OVX gilts treated with oil vehicle, estradiol or progesterone, was exposed to NAL. GnRH secretion increased in response to NAL and did not differ between the three steroid treatment regimens. This result is surprising in view of the importance given to the steroidal milieu in EOP regulation of GnRH/LH secretion *in vivo*. Alternatively, Barb *et al.* (1990) have provided evidence from *in vitro* porcine anterior pituitary cultures, that β-endorphin inhibits basal LH secretion, and that this is reversed by the addition of NAL. GnRH-stimulated and basal LH secretion were also increased when pituitary cell cultures were exposed to NAL, suggesting that locally produced pituitary opioids may act to suppress LH release from the anterior pituitary, independent of hypothalamic opioidergic regulation of GnRH secretion. Currently there is very little evidence as to whether the opioids function to inhibit GnRH release within the hypothalamus by acting pre-synaptically to inhibit stimulatory noradrenergic inputs, or post-synaptically, directly inhibiting the GnRH neuron. Possible interactions between the noradrenergic, opioidergic and GnRH neuronal systems are represented in Figure 2.1.

2.5.5 Norepinephrine

For many years the central noradrenergic system has been accepted as the main driving force of the GnRH pulse generator. As discussed earlier, it is probably not "the" pulse generator, but it is definitely one of the main modulators of GnRH secretion. Anatomically, GnRH cell bodies are located within the MBH and MPOA, with axons coursing through the hypothalamus, terminating in the ME at the hypophysial portal vessels (Leranth *et al.*, 1985a; Lehman *et al.*, 1986; Kineman *et al.*, 1988). Furthermore, many noradrenergic fibres, originating from the adrenergic (A) 1, A2 and A6 cell groups, originating in the nucleus accumbens, have been found in close proximity to GnRH cell bodies in the rat (Chen *et al.*,

FIGURE 2.1 Diagrammatic representation of the possible sites for noradrenergic and opioidergic regulation of GnRH secretion in the pig. ARC = arcuate nucleus; EOP = endogenous opioid peptides; GnRH = gonadotropin releasing hormone; LH = luteinizing hormone; ME = median eminence; MPOA = medial preoptic area; NE = norepinephrine.



1989b; Horvath *et al.*, 1992), ewe (Conover *et al.*, 1993; Lehman and Karsch, 1993) and pig (Leshin *et al.*, 1996).

From a physiological viewpoint, there are several reports of α_1-noradrenergic receptor mediated stimulation of GnRH secretion in the rat (Negro-Vilar *et al.*, 1979; Leung *et al.*, 1982; Nowak and Swerdloff, 1985; Condon *et al.*, 1989; Clough *et al.*, 1990; Jarry *et al.*, 1990; Leonhardt *et al.*, 1991) and primate (Kaufman *et al.*, 1985; Terasawa *et al.*, 1988; Gearing and Terasawa, 1991). Although it is known that α_1-receptors mediate the stimulatory effect of norepinephrine (NE), the hypothalamic site of action is relatively unknown. Negro-Vilar *et al.* (1979) demonstrated that in ME fragments from male rats incubated *in vitro,* high doses of NE stimulated GnRH release. Similarly, Clough *et al.* (1988) demonstrated that NE stimulated the *in vitro* release of GnRH from POA/AH explants obtained from steroid primed and OVX rats. Using push pull perfusion in OVX rhesus monkeys, Terasawa *et al.* (1988) correlated LH and GnRH pulsatility, and NE and GnRH pulsatility in the stalk median eminence. They noticed that NE pulses preceded GnRH pulses by an interval of approximately 10 minutes, and that there were extra NE pulses between those synchronous with GnRH. More recently, Jarry *et al.* (1990) have shown that NE in the POA/AH in OVX rats is also released in a pulsatile manner, however, they were unable to correlate these pulses with LH pulses. Also, in this study the use of the specific α_1-receptor antagonist, doxazosine, in the POA/AH eliminated LH pulsatility, but it did not when applied in the MBH.

NE stimulated GnRH release from male rat MBH *in vitro* in a dose dependent manner. This effect was blocked using the α-receptor antagonist, phentolamine, but not the ß-receptor antagonist, propranolol (PROP) (Nowak and Swerdloff, 1985). Similarly, *in vitro* addition of the α_1-receptor agonist, phenylephrine (PHEN), to the hypothalami of male rats stimulated release of GnRH (Leposavic *et al.*, 1990). *In vivo* in the rhesus monkey, Kaufman *et al.* (1985) found that blockade of α_1-receptors with phenoxybenzamine (PBZ) or prazosin (PRAZ) caused an inhibition of pulsatile LH secretion and fewer MUA volleys within the hypothalamus, whereas blockade of α_2-receptors with yohimbine had no effect on either parameter. This effect was also demonstrated during *in vivo* perfusion of the stalk median eminence of OVX rhesus monkeys, that addition of NE or methoxamine, an α_1-agonist, stimulated pulsatile GnRH secretion, and PRAZ suppressed but did not eliminate pulsatile GnRH secretion (Terasawa *et al.*, 1988). PRAZ was later shown to affect pulse amplitude but did not affect the frequency of GnRH pulses (Gearing and Terasawa, 1991). These results suggest that the stimulatory effects of norepinephrine on GnRH secretion are mediated by an α_1-receptor mechanism at the hypothalamic level.

The use of NE synthesis inhibitors has also demonstrated that a functional noradrenergic drive is important to continued GnRH secretion. Chang *et al.* (1992) found that in OVX estrogen-primed rats the NE synthesis inhibitors, AIMAX and DDC (dopamine-ß-hydroxylase inhibitor), both reduced MBH NE concentrations and peripheral LH concentrations. Kim *et al.* (1993) showed that incubation of hypothalamic tissue, obtained from male rats, in medium containing 6-hydroxy dopamine (6-OHDA), a NE neurotoxin, decreased NE content and also decreased GnRH mRNA level. This effect was reversed by the addition of NE to the medium. Furthermore, *in vitro* progesterone-stimulated GnRH

secretion and increased GnRH mRNA levels in tissue from estradiol-primed OVX rats treated with DDC, was suppressed compared with untreated controls; hypothalamic NE concentrations were also suppressed (Kim *et al.*, 1993 and 1994). These studies indicate that noradrenergic neurotransmission is involved in GnRH secretion and gene expression in the rat hypothalamus.

Conversely, the noradrenergic system, may in fact represent a permissive input to GnRH neurons and not be the main stimulatory influence, as interruption of the noradrenergic inputs, using receptor blockade, surgical deafferentation, neurotoxins and synthesis inhibitors, have provided some surprising results. Following castration in male rats, LH increased dramatically from day 1 to 40, however there were no associated increases in hypothalamic NE content or α-receptor number throughout this time period and testosterone replacement suppressed LH secretion but did not affect NE parameters (Herdon *et al.*, 1984). Neither acute inhibition of the NE system using α-antagonists PBZ or phentolamine, or the synthesis inhibitor, α-methyl-p-tyrosine (αMPT), or chronic depletion of hypothalamic NE using 6-hydroxydopamine (6-OHDA), had any effect on the long-term post-castration rise in LH secretion; however, there was an initial suppression of LH in response to these treatments, suggesting that a noradrenergic dependent system is not involved in the generation of pulsatile LH secretion following castration in the male rat (Herdon *et al.*, 1984). In a similar study, acute α-adrenergic blockade using PBZ inhibited pulsatile LH release in sham hypothalamic transected (HT), OVX rats but did not in HT, OVX rats. However, following chronic blockade of the noradrenergic input, induced by surgical deafferentation of the ascending noradrenergic fibres (HT), LH pulsatility was restored within 4 weeks of surgery (Clifton and Steiner, 1985). In a more recent study, Akema *et al.* (1990) report that following treatment with 6-OHDA, OVX rats maintained pulsatile LH secretion and that phentolamine was ineffective in blocking an estrogen induced LH surge. Furthermore, Leonhardt *et al.* (1991) administered 5-amino-2,4-dihydroxy-α-methylphenylethylamine (5-ADMP), a neurotoxin specific to NE, to OVX rats. Treatment resulted in a 98% depletion of POA/AH NE by 52 hours after the injection. Immediately after receiving 5-ADMP, LH secretion was inhibited, but by 4 hours after injection pulsatile LH release was again evident; at 52 hours, LH levels were identical to that of control rats. Administration of prazosin (PRAZ), an α_1-receptor blocker, did not inhibit LH release in treated rats, but did in controls. As NE inhibition can be mediated via ß-receptors, PROP, the ß-antagonist, was administered with 5-ADMP in some rats; this treatment did not prevent the transient decrease in LH secretion, indicating that there was no massive release of NE in response to 5-ADMP which could have inhibited LH secretion (Leonhardt *et al.*, 1991). These studies indicate that NE allows LH pulsatility, acutely, but that another regulatory system is functional chronically, in the absence of NE, further suggesting that the GnRH neurons are driven by more than one pulse generator, a NE-dependent and a NE-independent system(s). Normally the NE-dependent system is the dominant stimulatory system, but in the case of the surgical blockade, a chronic interruption of the NE system causes the NE-independent system to activate (Clifton and Steiner, 1985).

Although generally considered a stimulatory input to GnRH neurons, NE has both inhibitory and excitatory effects on GnRH/LH secretion, dependent on the circulating steroid concentration (Taleisnik and Sawyer, 1986). In a study by Parvizi and Ellendorff (1982) in

OVX Göttingen miniature pigs, NE exerted a dual effect on LH secretion dependent on the dose and the hypothalamic site to which it was applied. In OVX rats i.c.v. infusion of NE, PHEN, the ß-receptor agonist, isoproterenol (ISO), or the α_2-agonist, clonidine, significantly reduced LH secretion (Leung *et al.*, 1982). However, in OVX steroid-primed rats infused with NE, PHEN or clonidine, LH secretion was stimulated and ISO had no effect. This study demonstrates that inhibitory effects of NE can be mediated by both α- and ß-receptors in an environment devoid of steroids, but that stimulatory actions are mediated by α-receptors. In a series of similar studies, it was found that NE inhibitory effects on GnRH/LH release are mediated by ß-adrenergic receptors located within the premammillary nuclei, and that inhibitory effects of locus ceruleus stimulation on GnRH secretion could be prevented by pretreatment with PROP or by premammillary lesions (Caceres and Taleisnik, 1980 and 1982; Dotti and Taleisnik, 1984). In a study using dispersed pig pituitary cells from prepubertal male and female animals, it was found that NE inhibited GnRH stimulated release of LH secretion but that this action was prevented by addition of PROP, indicating a ß-receptor mediated inhibition of LH release (Li, 1989). In a recent study, Condon *et al.* (1989) found that the α1-agonist, methoxamine, was excitatory to GnRH neurons in the ARC of female guinea pigs, and that ISO was inhibitory to a population of these ARC neurons. A greater number of neurons were excited by methoxamine following an increase in serum estrogen levels. These results indicate that NE excitatory effects are mediated by α_1-receptors and the inhibitory effects are mediated by ß-receptors.

The exception to the α_1-receptor mainly acting in a stimulatory capacity, is the ovary intact, seasonally anestrous, ewe. Phenoxybenzamine injected into the POA increased mean LH concentrations and LH pulse frequency, although it had no effect at the level of the ME (Havern *et al.*, 1991), suggesting that in the anestrous ewe, an α-receptor mediated mechanism functions to inhibit GnRH secretion at the level of GnRH cell bodies and not at the terminals. Goodman (1989) showed that during seasonal anestrus, the noradrenergic system stimulates an inhibitory dopaminergic input to the GnRH neurons, thereby causing the apparent inhibition of GnRH by NE. In breeding season, an α-mediated inhibitory effect on GnRH secretion, similar to that in anestrous ewes, is thought to mediate some of the effects of estradiol negative feedback on GnRH secretion, and affect GnRH pulse amplitude (Goodman *et al.*, 1995; Goodman *et al.*, 1996). Overall, however, there is a vast body of conclusive evidence which shows that the central noradrenergic system stimulates GnRH secretion via an α-noradrenergic receptor.

2.5.6 Noradrenergic and Opioidergic Interactions Regulating GnRH Secretion

Several studies in the rat suggest that the EOP inhibit noradrenergic inputs to GnRH neurons (for reviews see Grossman and Dyer, 1989; Kalra *et al.*, 1989). Akabori and Barraclough (1986) showed that LH secretion and NE concentration and turnover rates decreased in the MPOA of morphine treated rats. Furthermore, morphine, ß-endorphin and met-enkephalin inhibited electrically stimulated release of ^{3}H-noradrenaline from perfused slices of rat preoptic area; the effects of morphine and ß-endorphin were reversed by the addition of NAL (Diez-Guerra *et al.*, 1987). Dyer and Grossman (1988) demonstrated that

neurons in the MPOA which were excited by ventral noradrenergic tract stimulation were inhibited when morphine was applied to the area, and that morphine and NAL in combination prevented this inhibition. Similarly, opioidergic blockade using NAL increased hypothalamic MUA volleys and LH pulsatility, but administration of the α_1-antagonist, phenoxybenzamine, abolished both electrical activity and LH pulsatility (Nishihara *et al.*, 1991). Dyer *et al.* (1991) also found that opioidergic binding and norepinephrine content in the POA/AH of OVX estrogen-primed rats decreased following electrolytic and neurochemical lesioning of the ventral noradrenergic tract. Together these results indicate that the EOP and noradrenergic neuronal systems interact to regulate GnRH/LH secretion.

Several lines of evidence support an alternative mechanism for noradrenergic/opioidergic interactions regulating GnRH secretion. Miller *et al.* (1985) found that NAL still stimulated LH release in male rats in which the ascending noradrenergic tract had been cut. Phenoxybenzamine was ineffective in preventing the NAL-induced rise in LH in the lesioned rats but prevented it in sham lesioned rats, indicating that the EOP do not require a functional noradrenergic system to inhibit GnRH secretion. Furthermore, NAL and phenylephrine administered concomitantly had a potentiated action on GnRH release from rat POA-MBH *in vitro*, compared with either drug administered separately (Clough *et al.*, 1990). These authors suggest that the independent effects of both the opioid and noradrenergic compounds indicate that EOP and NE systems regulate GnRH neurons independently.

Arvidsson *et al.* (1995) and Ding *et al.* (1996) identified post-synaptic μ-opioid receptors in the MPOA and anterior hypothalamus of rats, and the GnRH neurons of guinea pigs possess functional μ-opioid receptors (Lagrange *et al.*, 1995). When these receptors are activated, they may function to reduce the receptivity of the post-synaptic neuron to incoming stimuli (Leranth *et al.*, 1995; Ding *et al.*, 1996). Pre-synaptic μ-receptors when activated may also inhibit neurotransmitter release from the neuron (Ding *et al.*, 1996). Both of these mechanisms have been suggested as mechanisms whereby the EOP could inhibit GnRH secretion.

2.5.7 Noradrenergic Regulation of GnRH Secretion in the Pig

In the pig there is a paucity of evidence regarding the noradrenergic regulation of the GnRH pulse generator. However, the anatomical basis for interactions between the noradrenergic and GnRH systems exist in the pig. Leshin *et al.* (1989 and 1996) have identified tyrosine hydroxylase and dopamine-ß-hydroxylase immunopositive neurons in the MPOA, in the same area as GnRH immunopositive neurons were identified by Kineman *et al.* (1988). Early studies by Parvizi and Ellendorff (1978 and 1982) demonstrated that the noradrenergic system may be involved in the regulation of LH secretion in the Göttingen miniature pig. NE was administered into the central ventricular system and microinjected into discrete hypothalamic nuclei, where it elicited either stimulatory or inhibitory LH responses, that were dose dependent and location specific. The administration of the noradrenergic synthesis inhibitors, DDC (Chang *et al.* 1990) and AIMAX (N-methyl-N'[1-methyl-2-propenyl]1,2 hydrazine-dicarbothioamide) (Kesner *et al.*, 1987; Chang *et al.*, 1993a), suppressed mean and basal LH secretion and prevented the estradiol induced LH surge in

OVX gilts. This was established to be mediated at the level of the hypothalamus, as a regimen of pulsatile exogenous GnRH reestablished LH pulsatility in the AIMAX treated gilts (Kesner *et al.*, 1987). It appears that a noradrenergic input is important to maintain basal and surge secretion of LH in the OVX gilt.

There is even less evidence regarding noradrenergic, opioidergic and GnRH interactions in the female pig. A role for opioidergic inhibition of GnRH/LH secretion in the pig has been firmly established, as previously discussed. However, opioidergic effects on systems impinging on GnRH or directly on GnRH neurons are little understood. Further anatomical evidence provides the basis of an interaction between the three systems, as GnRH, POMC and dopamine-ß-hydroxylase immunopositive neurons have all been identified within the MPOA of the porcine hypothalamus (Kineman *et al.*, 1988 and 1989; Leshin *et al.*, 1996). Chang *et al.* (1993a) suggest that the EOP inhibit GnRH secretion presynaptically, via inhibition of the noradrenergic system, based on the inability of NAL to stimulate LH secretion in AIMAX treated gilts. However, the true nature of these interactions awaits further examination.

2.5.8 Gamma-Aminobutyric Acid

GABA is considered to be the major central inhibitory neurotransmitter, although both stimulatory and inhibitory actions on GnRH and LH secretion have been reported. These actions are mediated by $GABA_A$ and $GABA_B$ receptor types (Bergen *et al.*, 1991) and are also dependent on the steroidal environment of the animal and the experimental protocol utilized. However, a stimulatory role for GABA in GnRH secretion is more equivocal, and will not be fully considered here.

It is thought that GABA may mediate estradiol negative feedback on the GnRH neuronal system (Mansky *et al.*, 1982; Herbison *et al.*, 1989 and 1990), as GABAergic neurons are known to concentrate estradiol (Flugge *et al.*, 1986) and have been shown to synapse directly with GnRH cell bodies in the MPOA (Leranth *et al.*, 1985b). Several studies involving GABA agonists and antagonists have demonstrated a role for GABA in the inhibitory regulation of GnRH and LH secretion. Bergen *et al.* (1991) determined that exposure of MPOA GnRH neurons to the $GABA_B$ agonist baclofen (BAC) decreased levels of GnRH mRNA, and that muscimol (MUS; $GABA_A$ agonist) had no significant effect, and bicuculline (BIC; $GABA_A$ antagonist) decreased mRNA levels. These results suggest that activation of $GABA_B$ receptors decreases GnRH synthesis as well as release, and that $GABA_A$ receptors may mediate a stimulation of GnRH synthesis, perhaps indirectly, as MUS had no direct effect. In other studies, both BAC and MUS prevented the estradiol benzoate induced LH surge and increased the interval between MUA volleys and LH pulses in OVX rats (Adler and Crowley, 1986; Akema and Kimura, 1991; Kimura *et al.*, 1993). Alternatively, Masotto *et al.* (1989) found that administration of GABA or MUS to ME fragments *in vitro* stimulated GnRH release, which was blocked by BIC and that BAC had no effect on basal GnRH release.

In addition to the studies which have utilized a purely pharmaceutical approach, others have investigated GABA metabolism within the hypothalamus in relation to GnRH and LH

secretion rates. Administration of GABA transaminase inhibitor, elevated MPOA concentrations of GABA, resulting in a blockade of the LH surge in intact female rats (Donoso and Banzan, 1986), the estradiol induced LH surge in OVX rats (Seltzer and Donoso, 1992) and a suppression of the acute post-castration rise in LH secretion in male rats (Donoso, 1988). Using push pull perfusion, Jarry *et al.*, (1988) showed that GABA concentrations in the PO/AH area decrease immediately prior to the onset of an LH pulse in OVX rats, suggesting an inverse relationship between GABA and LH secretion. These pulses of LH could be inhibited by intrahypothalamic application of GABA. Furthermore, application of BIC blocked pulsatile LH, indicating that GnRH neurons must first be inhibited by GABA prior to a disinhibition which would result in synchronous GnRH release (Jarry *et al.*, 1991). In contrast to these findings, Herbison *et al.* (1991a) did not find a correlation between the timing of decreased extracellular GABA concentrations and the initiation of LH pulses but also noted that application of GABA and BIC were able to completely abolish LH pulsatility. These authors suggested that oscillations in GABA concentration are necessary for pulsatile GnRH and LH release and that in the total absence of GABA tone, there is a reduction in GnRH/LH pulsatility.

In the GT1-1 cell line, MUS ($GABA_A$ agonist) and GABA infusions resulted in a rapid depolarization of the cell and release of GnRH, while BAC ($GABA_B$ agonist) inhibited pulsatile release of GnRH (Martinez de la Escalera *et al.*, 1994). Similarly, application of GABA to GT1-7 cells, another immortalized GnRH cell line, resulted in excitation of the cells, reversible by applying BIC ($GABA_A$ antagonist) (Hales *et al.*, 1994). *In vitro*, BAC hyperpolarized GnRH neurons from OVX guinea pigs (Lagrange *et al.*, 1995). The results from these studies demonstrate that immortalized cell lines and GnRH neurons express functional GABA receptors, and furthermore, that inhibitory actions of GABA on GnRH secretion are mediated by $GABA_B$ receptors, at the level of the MPOA, and that the majority of the GnRH stimulatory responses to GABA are mediated at the level of the ME and ARC by $GABA_A$ receptors. Stimulatory responses may represent an indirect action of GABA on another inhibitory neural input to the GnRH system, thus disinhibiting GnRH secretion.

2.5.9 Gamma-Aminobutyric Acid and Opioidergic Interactions

Studies have shown that GABA may also interact with the EOP system to regulate GnRH secretion. Activation of either $GABA_A$ or $GABA_B$ receptors abolished NAL induced LH release in the male rat and the steroid primed OVX rat (Masotto and Negro-Vilar, 1987; Brann *et al.*, 1992). These mechanisms are mediated centrally, not at the pituitary level, as administration of BAC or MUS had no effect on exogenous GnRH-induced release of LH (Brann *et al.*, 1992). Furthermore, administration of amino-oxyacetic acid, which increases brain GABA concentrations by inhibiting GABA catabolism, also abolished the LH response to NAL (Brann *et al.*, 1992). *In vitro*, MUS stimulated GnRH from ME fragments, as did NAL; together there was an additive effect of NAL and MUS on GnRH secretion (Masotto *et al.*, 1989), which may indicate that activation of the $GABA_A$ receptor further disinhibits GnRH secretion from an inhibitory EOP input. It was implied in these studies that the EOP and GABA systems may actually be impinging on the stimulatory noradrenergic inputs to the

GnRH system, although these studies failed to clearly demonstrate this (Masotto *et al.*, 1989; Brann *et al.*, 1992; Kimura *et al.*, 1993). Recently, in contrast to these findings, Jarry *et al.* (1995) found that there was no change in MPOA GABA concentrations in response to intrapreoptic application of either ß-endorphin or NAL, although LH secretion was affected, suggesting that the two systems may not interact to regulate GnRH secretion.

2.5.10 Gamma-Aminobutyric Acid and Noradrenergic Interactions

Other studies have shown that GABAergic regulation of GnRH secretion may involve interactions with the stimulatory noradrenergic inputs to GnRH neurons. Several studies suggest that GABA inhibits GnRH secretion via a presynaptic inhibition of noradrenergic inputs to the GnRH neuron. Glutamate decarboxylase immunoreactive neurons, putative GABAergic neurons, and high affinity GABA binding sites have been visualized in the locus ceruleus and dorsal and ventral noradrenergic tracts which are believed to innervate GnRH neurons (Palacios *et al.*, 1981; Berod *et al.*, 1984). In OVX rats, i.c.v. administration of MUS decreased NE turnover in the MPOA and decreased serum LH concentrations (Fuchs *et al.*, 1984). Adler and Crowley (1986) found that the GABA agonists, MUS and BAC, prevented the estradiol benzoate induced LH surge, and furthermore, decreased NE concentrations and turnover rates in the MPOA and MBH. Mansky *et al.* (1982) found that estrogen administration reduced LH secretion, and concomitantly increased GABA, while decreasing NE release and turnover rates within the hypothalamus. These results further support the role for GABA mediating the effects of estrogen negative feedback on GnRH and LH secretion.

Based on anatomical data from Leranth *et al.* (1988b) which shows noradrenergic synapses on GABA and GnRH neurons in the MPOA of the rat, an alternative hypothesis for GABA/NE interactions regulating GnRH secretion has been suggested by Herbison *et al.* (1989 and 1990). This group have shown that both basal and KCl stimulated GABA release from MPOA slices *in vitro* was greater from estrogen-primed OVX rats than unprimed OVX rats. Furthermore, GABA release could be stimulated from these MPOA slices by the addition of NE to the perfusion medium, and that tissue from the estrogen-primed rats released more GABA in response to NE (Herbison *et al.*, 1989). Stimulation of the A1 noradrenergic neurons in estrogen-primed OVX rats resulted in increased concentrations of both NE and GABA in hypothalamic dialysates (Herbison *et al.*, 1990). These results led this group to postulate that there is an excitatory noradrenergic input to GABA neurons which is modulated by circulating estrogen concentrations, and that this interaction is responsible for mediating the effects of estrogen on GnRH and LH secretion throughout the estrous cycle.

Conversely, GABA may function independently of the NE system. Animals treated with i.c.v. injections of 6-hydroxydopamine (6-OHDA), which destroys hypothalamic NE content, still maintained pulsatile LH secretion. This was shown not to have been due to remaining NE terminals, as phentolamine (α-adrenergic receptor antagonist) could not suppress the estradiol benzoate induced LH surge in these rats. However, in 6-OHDA treated rats MUS was still able to inhibit pulsatile LH secretion and the estradiol benzoate induced

LH surge (Akema *et al.*, 1990). Hartman *et al.* (1990) found that administration of the GABA antagonists, BIC and phaclofen ($GABA_B$) enhanced NE stimulated GnRH/LH secretion from estrogen-primed OVX rats . In another study by Akema and Kimura (1993), MUS and BAC administered i.c.v. to estrogen-primed OVX rats inhibited the stimulatory effect of exogenous NE on LH secretion. These results indicate that GABA and NE are organized in parallel, both systems having inputs directly on the GnRH neuron. Activation of either GABA receptor type may function to reduce the responsiveness of the post-synaptic neuron (GnRH) to stimulatory inputs (NE). Possible interactions between the GABAergic, noradrenergic and opioidergic systems regulating GnRH secretion are represented in Figure 2.2.

2.5.11 Excitatory Amino Acids

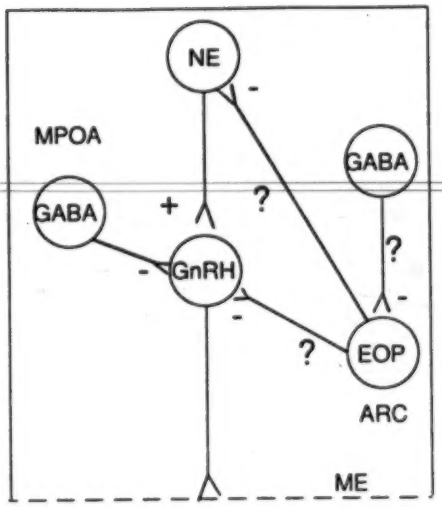
Excitatory amino acids (EAA), such as aspartate and glutamate, are ubiquitous neurotransmitters in the mammalian brain (Mayer and Westbrook, 1987). Glutamate has been localized in presynaptic boutons throughout the suprachiasmatic, ventromedial, arcuate and paraventricular nuclei in the rat hypothalamus, and aspartate immunoreactivity has been identified in the medial hypothalamus (van den Pol, 1991). These EAA function via two receptor types, ionotropic, which activate ion channels, and metabotropic, which are G-protein linked. The ionotropic receptors, which mediate the GnRH/LH secretory responses, are subclassified as N-methyl-D-aspartate (NMDA), kainate and D,L-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type receptors based on their respective selective agonists (as reviewed by Brann and Mahesh, 1994).

Several studies have demonstrated that administration of NMDA stimulates LH secretion (Brann and Mahesh, 1991a and 1991b; Saitoh *et al.*, 1991; Lee *et al.*, 1993; Luderer *et al.*, 1993). These stimulatory effects of NMDA on LH secretion appear to require an estrogenic environment (Estienne *et al.*, 1990b) as there is either no effect, or an inhibition of LH secretion in response to treatment in unprimed OVX animals (Estienne *et al.*, 1990b; Reyes *et al.*, 1990; Brann and Mahesh, 1992; Luderer *et al.*, 1993). Furthermore, the effect of NMDA on LH secretion has been shown to be centrally mediated, as LH secretion from pituitary cultures from both monkeys and rats did not increase in response to the addition of NMDA (Tal *et al.*, 1983). In a more recent study, NMDA and kainic acid (kainate receptor agonist) both stimulated LH secretion but also increased cytoplasmic GnRH mRNA levels in the POA/AH of male rats (Gore and Roberts, 1994).

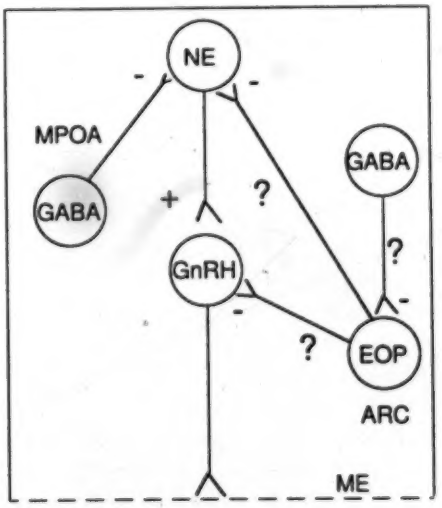
NMDA stimulated LH release in estradiol primed OVX ewes but not in OVX ewes (Estienne *et al.*, 1990b) and blockade of the NMDA receptor with D,L-2-amino-5-phosphonovaleric acid (AP5), an NMDA receptor antagonist, did not suppress LH secretion in OVX lambs (Hileman *et al.*, 1992). Hileman *et al.* (1992) questioned the involvement of EAA in the generation of GnRH/LH pulses in the female sheep, as interruption of EAA neurotransmission with AP5 did not prevent pulsatile LH secretion. Further evidence for the importance of estradiol in mediating a stimulatory response to NMDA was provided by Luderer *et al.* (1993) who showed that NMDA increased LH secretion in metestrous and proestrous rats but decreased LH secretion in OVX rats.

FIGURE 2.2 Diagrammatic representation of the possible interactions between the GABAergic, noradrenergic and opioidergic systems regulating GnRH secretion, as discussed in this literature review. ARC = arcuate nucleus; EOP = endogenous opioid peptides; GABA = gamma-aminobutyric acid; GnRH = gonadotropin releasing hormone; ME = median eminence; MPOA = medial preoptic area; NE = norepinephrine.

a) parallel



b) series



Unlike the monkey (Pohl, 1991) and the pig (Sesti and Britt, 1992, 1993 and 1994), during lactation in the rat, NMDA administration does not stimulate LH secretion (Pohl *et al.*, 1989). Both NMDA and kainate failed to stimulate LH secretion in lactating rats suckling 8 pups; however, rats suckling 2 pups responded to treatment with NMDA and kainate (Abbud and Smith, 1993). Studies by both Pohl *et al.* (1992) and Abbud and Smith (1993), in which lactating rats were ovariectomized or treated with the progesterone antagonist, RU 486, failed to stimulate LH secretion after NMDA treatment. The authors suggest that the intensity of the suckling stimulus and not the steroidal milieu affects the response to NMDA during lactation, and that the suckling stimulus may alter the GnRH neuron's responsiveness to stimulatory and inhibitory neural inputs.

The NMDA inhibition of LH secretion from OVX rhesus monkeys appears to involve the inhibition of GnRH by corticotropin releasing hormone (CRH), and perhaps opioids, as plasma cortisol was elevated following NMDA administration (Reyes *et al.*, 1990). Naloxone pretreatment in young male mice greatly potentiated the LH response to NMDA treatment (Miller and Gibson, 1994). Saitoh *et al.* (1991) showed that following NMDA administration *c-fos* protein, a marker for neural activation, could be located in identified noradrenergic, CRH, and ß-endorphin neurons but not GnRH neurons. In a similar study, Lee *et al.* (1993) showed that although i.c.v. and i.v. injections of NMDA could stimulate LH secretion and *c-fos* expression in an area of the hypothalamus containing GnRH neurons, the GnRH neurons themselves did not express *c-fos*. The results from these studies indicate that GnRH neurons do not possess EAA ionotropic receptors, and that responses to NMDA are likely modulated by other neural inputs to the GnRH system.

Although these studies demonstrate that EAA are capable of eliciting a GnRH/LH response in steroid primed animals, is there a physiological role for these EAA? Ping *et al.* (1994), using *in vivo* microdialysis, have shown that aspartate and glutamate concentrations increase in the MPOA of estrogen primed OVX rats, immediately prior to peak serum LH concentrations in response to a progesterone induced LH surge. Furthermore, administration of MK801, an NMDA receptor antagonist, on proestrous, prevented the LH surge and ovulation (Luderer *et al.*, 1993) and i.c.v. infusion of AP5, an NMDA receptor antagonist, and DNQX, a kainate receptor antagonist, prevented the steroid induced LH surge (Brann and Mahesh 1991a and 1991b) and interrupted pulsatile secretion of LH in OVX rats (Ping *et al.*, 1994b). Bourguignon *et al.* (1995) have demonstrated that when endogenous glutamate synthesis is impaired, GnRH pulsatility is suppressed in hypothalamic explants from male rats. The study by Saitoh *et al.* (1991) indicated that the greatest amount of *c-fos* expression occurred in noradrenergic neurons, identified by many as the primary stimulatory input to GnRH neurons (as reviewed by Kalra and Kalra, 1983). In fact, Navarro *et al.* (1994) have recently shown that both NMDA and kainate receptors mediate the release of ^{3}H-noradrenaline from rat MBH slices *in vitro*. These studies indicate, at least in the rat, that EAA may play an important role in the generation of the LH surge and subsequent ovulation.

2.5.12 Excitatory Amino Acid Regulation of GnRH and LH Secretion in the Pig

In OVX and progesterone treated OVX gilts, NMDA inhibited LH secretion, but LH

secretion was not affected in estrogen treated OVX gilts (Barb *et al.*, 1992b; Chang *et al.*, 1993b). In the OVX and progesterone treated animals it is possible that the EAA stimulated the release of an inhibitory hypothalamic factor, which inhibited GnRH secretion and thus, LH secretion in this study. Barb *et al.* (1992b) suggested that the opioidergic system, known to be inhibitory to LH secretion (Barb *et al.*, 1985 and 1986a), could be responsible for this action. In a study designed to test this hypothesis, Chang *et al.* (1993b) administered NMDA or NMDA in combination with NAL to OVX and progesterone treated OVX gilts. As in the previous study, NMDA inhibited LH secretion in all gilts and NAL failed to reverse this inhibition, indicating that the EOP do not appear to be involved in the NMDA mediated suppression of LH secretion. Contrary to these findings, Estienne *et al.* (1995) have shown that a high dose (10 mg/kg) of NMDA increases LH concentrations in prepubertal gilts, which is quite unexpected as circulating steroids in these animals would be low, and if they are compared to the OVX gilts in Barb's study, a decrease in serum LH concentrations would be expected.

Using a different physiological model, administration of NMDA to lactating sows and estrogen primed OVX gilts significantly increased LH secretion (Sesti and Britt, 1992, 1993 and 1994). This was demonstrated to be a hypothalamic effect and not a pituitary effect on LH secretion, as gilts which had been passively immunized against GnRH did not respond to treatment with NMDA (Sesti and Britt, 1992). These authors suggest that NMDA only stimulates LH secretion when secretion of GnRH and LH are already inhibited. NMDA elicited LH responses which increased in a linear fashion as lactation progressed, suggesting that releasable pools of GnRH increased as lactation progressed (Sesti and Britt, 1993). Circulating estradiol concentrations do not seem to effect the LH response to NMDA, as lactating sows, which do not have measurable levels of this steroid (Edwards, 1982), respond to treatment with NMDA.

As well as a hypothalamic effect, Barb *et al.* (1993) found that NMDA added to pituitary cell cultures from OVX, follicular phase and luteal phase gilts directly stimulated LH release from the gonadotropes. This effect was reversible only in the follicular phase cultures by addition of the NMDA antagonist, AP5. As NMDA is a non-specific agonist which stimulates the release of many other anterior pituitary hormones (Barb *et al.*, 1992b), perhaps NMDA functions via an indirect effect on one of these hormones to affect LH secretion at the level of the pituitary. Unlike the rat, a physiological role for EAAs in the regulation of GnRH and LH secretion has yet to be identified in the pig.

2.5.13 Dopamine

Dopaminergic neurons innervating the hypothalamus originate in the cell groups known as A11, A12, A13 and A14. The A12 group represent the tuberoinfundibular dopamine (DA) neurons which represent a large number of cell bodies within the arcuate nucleus of the hypothalamus (as reviewed by Weiner *et al.*, 1988). DA released into the hypophysial portal vessels is largely responsible for the regulation of PRL secretion from the pituitary (as reviewed by Ben-Jonathan, 1985), but has also been suggested by many as a regulator of GnRH and LH release in several species. Negro-Vilar *et al.* (1982) reported that

in the ME, DA content is correlated with LH pulses, suggesting that the tuberoinfundibular dopaminergic system could replace the noradrenergic stimulatory system when NE is chronically blocked or depleted. However, studies regarding the dopaminergic regulation of GnRH/LH secretion have largely resulted in conflicting views. Most of the evidence, as reviewed by Kalra and Kalra (1983) and Fink (1988), suggest that DA is inhibitory to LH secretion, via inhibition of hypothalamic GnRH secretion, but there are also reports of a stimulatory influence on GnRH secretion.

As with many neurotransmitters, the effect that DA can have is influenced by circulating steroid levels. Judd *et al.* (1979) reported that DA had a stronger inhibitory action in agonadal women than in normal women, and that when agonadal women had steroid replacement the inhibitory effect of DA on LH secretion was lessened. Administration of DA to human MBH *in vitro* stimulated basal GnRH release; the addition of haloperidol, the D_2-receptor antagonist, on its own had no effect on GnRH release. However, the concomitant administration of haloperidol and DA prevented the DA stimulated GnRH release previously demonstrated, while concomitant administration of phentolamine, the noradrenergic antagonist, did not (Rasmussen *et al.*, 1986). Alternatively, the infusion of fenoldopam, a D_1-receptor agonist, had no effect on basal or pulsatile LH release during the follicular phase of the menstrual cycle in normal women, but fenoldopam did increase the LH response to exogenous GnRH administration (Boesgaard *et al.*, 1991). In contrast to these results, Andersen *et al.* (1987) demonstrated that a physiological dose of DA inhibited basal LH secretion in normal women during the follicular phase of the menstrual cycle. This suppression appeared to be mediated via a D_2 receptor mechanism, as the effect of DA was reversible using the D_2 antagonist, metoclopramide. These results suggest that in normal women, with measurable circulating estradiol concentrations, DA stimulates GnRH secretion from the hypothalamus via a D_2-receptor mediated mechanism and that DA may also affect pituitary sensitivity to GnRH via a D_1-receptor mediated mechanism.

The D_2 antagonist, metaclopramide, decreased GnRH neuronal electrical activity (MUA volleys) for two hours following administration to OVX rhesus monkeys, suggesting a stimulatory role for DA in the regulation of the pulse generator (Kaufman *et al.*, 1985). In other studies, haloperidol administration to proestrous rats has been shown to prevent the LH surge, the effect of which could be overcome by administration of CB-154 (Krieg and Cassidy, 1984). More recently, Li and Pelletier (1992a and 1992b) have shown that GnRH mRNA levels are positively regulated by DA, as bromocriptine increased message, and haloperidol decreased message in the hypothalamus of intact male and female rats. Furthermore, DA stimulated GnRH release from MBH fragments *in vitro* from intact male rats, but did not affect release from the tissue of castrated male rats. These effects were reversible using the DA antagonists, pimozide and haloperidol (Rotsztejn *et al.*, 1977). Rotsztejn *et al.* (1977) also found that DA did not affect GnRH secretion from tissue containing the more rostral structures of the hypothalamus, such as the organum vasculosum of the lamina terminalis (OVLT). These results suggest a stimulatory role for DA in the release of GnRH from axon terminals in the MBH and ME, but not at the level of the GnRH cell bodies. This corroborates evidence which shows the MBH and ME contain many DA neurons and DA receptors, and the area where interactions between GnRH and DA neurons

have been identified (McNeill and Sladek, 1978; Ajika, 1979; Leibowitz *et al.*, 1982; Spencer *et al.*, 1985; Kuljis and Advis, 1989).

Tasaka *et al.* (1985) report no effects of DA on pituitary tissue from intact female rats *in vitro*, however when the pituitary and MBH were perfused in series, DA suppressed LH secretion; addition of haloperidol, a D_2-receptor antagonist, to the media prevented the suppression. These results indicate that DA acts at the hypothalamic level, via a D_2 receptor mechanism, to suppress GnRH secretion and not directly at the pituitary level. Several studies in the anestrous ewe, have identified DA as the major inhibitory factor of GnRH/LH secretion during long days, mediating the effects of estradiol negative feedback (Legan *et al.*, 1977). DA inhibits pulsatile LH secretion by acting on the axons and terminals of GnRH neurons in the MBH, as implantation of pimozide, the D_2-receptor antagonist, in the retrochiasmatic area and ME prevents the suppression of LH secretion in the anestrous ewe (Havern *et al.*, 1991). Administration of the neurotoxin, 6-OHDA, destroyed only 20 percent of DA neurons in the A15 nucleus, a DA cell group identified only in sheep, but resulted in a threefold increase in pulsatile LH secretion in OVX estradiol treated ewes (Thiéry *et al.*, 1989). Similarly, administration of sulpiride, a D_2-receptor antagonist, to rams during long days significantly increased mean LH concentration and pulse frequency (Tortonese and Lincoln, 1994). Conversely, the D_2 agonist, bromocriptine, had no effect during long days, but this was likely due to the already suppressed circulating levels of LH (Tortonese and Lincoln, 1994). Havern *et al.* (1994) have recently demonstrated that lesioning of the A14 and A15 dopaminergic cell groups decreased, but did not completely abolish, estradiol inhibition of LH secretion during long days. This suggests that another system may be involved in the mediation of the estradiol negative feedback on GnRH/LH secretion during long days in the sheep.

There appear to be many species differences in the dopaminergic regulation of GnRH secretion. Other factors which determine the effect of DA on GnRH secretion appear to be the location within the hypothalamus and the circulating concentration of estrogen or testosterone. However it does appear that most of the stimulatory effects occur within the MBH and ME, at the level of the GnRH axons and terminals, and when circulating estrogen concentrations are high. Furthermore, DA is effective in the hypothalamus via D_2 receptors and may affect the pituitary directly, via D_1 receptors.

2.5.14 Neuropeptide Y

Neuropeptide Y (NPY) is a member of the pancreatic polypeptide family which has been identified in the MPOA, periventricular nucleus, anterior hypothalamus, and throughout the MBH, including the ARC and ME, of rats (Chronwall *et al.*, 1985). NPY immunoreactivity has also been colocalized with noradrenergic fibres (Everitt *et al.*, 1984), and synaptic contacts between NPY neurons and GnRH neurons have been identified within the preoptic nucleus of rats (Tsuruo *et al.*, 1990). Evidence suggests that NPY functions both as a neuromodulator of LH release, at the level of the pituitary, and as a neurotransmitter, having direct stimulatory and inhibitory actions on hypothalamic GnRH release. The conflicting effects of NPY on GnRH secretion at the hypothalamic level are

mediated via two receptor types, NPY_1, which mainly mediates stimulatory responses (some inhibitory), and NPY_2, which mediates inhibitory responses (Sheikh *et al.*, 1989; Wahlestedt *et al.*, 1990; Kalra *et al.*, 1992). The effect that NPY has on GnRH and LH secretion is also dependent on the steroidal milieu (Sabatino *et al.*, 1989 and 1990; Urban *et al.*, 1996).

NPY effectively stimulated GnRH release from ME fragments *in vitro* only when OVX rats had been treated with estradiol; higher doses of estrogen elicited a greater GnRH secretory response from NPY (Sabatino *et al.*, 1989 and 1990), suggesting a physiological relevance to NPY stimulatory effects during the estrous cycle and during the LH surge. Using push-pull perfusion, Woller and Terasawa (1992) found that infusion of NPY into the stalk median eminence of OVX and OVX estrogen-primed rhesus monkeys resulted in increased GnRH release. Although the results from the unprimed animals tend to contradict the findings in rats (Crowley and Kalra, 1987; Sabatino *et al.*, 1989), they did find that GnRH release was greater in the steroid primed animals. Similarly, NPY was only effective in stimulating GnRH release from MBH or ME tissue *in vitro* from intact male rats, or testosterone-treated castrates (Urban *et al.*, 1996). Recent studies have defined a potential physiological role for NPY regulation of GnRH secretion in the rat. Sahu *et al.* (1994) found that MBH NPY mRNA levels increased dramatically immediately prior to the estrogen induced gonadotropin surge in OVX rats. Furthermore, immunoneutralization of NPY blocked the steroid induced LH surge (Wehrenberg *et al.*, 1989) and LH pulsatility (Xu *et al.*, 1993 and 1996) in OVX rats, indicating a potential physiological role for hypothalamic NPY in both the pulse and surge generating mechanisms in the OVX rat.

In physiological states where estradiol is low and metabolic demands are increased, such as lactation, NPY mRNA is increased in the hypothalamus and may mediate some of the inhibitory effects on GnRH secretion (Smith, 1993). McShane *et al.* (1992 and 1993) found that i.c.v. administration of NPY inhibited the release of LH, and that NPY concentrations in the cerebro-spinal fluid and hypothalamic NPY mRNA levels were increased in feed restricted OVX ewes, compared with well fed controls. Malven *et al.* (1992) also report that injection of NPY at five different intraventricular locations resulted in the suppression of LH secretion in OVX ewes. These results would suggest that NPY may well be involved in the central mediation of nutrition-reproduction interactions.

Other neuroendocrine systems have been implicated in mediating the inhibitory effects of NPY on GnRH secretion. I.c.v. administration of NPY to OVX monkeys suppressed LH secretion (Kaynard *et al.*, 1990) and increased CRH concentrations within the hypothalamus (Haas and George, 1987), prompting the theory that NPY may stimulate the release of CRH which activates EOP systems to inhibit GnRH release. However, Xu *et al.* (1993) found that administration of NAL prevented NPY inhibition of LH secretion, but that treatment with a CRH antagonist was ineffective in reversing the inhibitory effects of NPY. These data suggest that the inhibitory effects of NPY may be due to the stimulation of EOP neurons but not CRH neurons. Furthermore, the presence of estrogen may either decrease the ability of NPY to stimulate opioidergic neurons, suppress the action of opioids on GnRH neurons, or activate a system which overrides the combined effects of NPY and opioids on GnRH secretion.

As well as functioning as a central neurotransmitter, NPY may have a

neuromodulatory function in the regulation of LH secretion. Studies have shown that NPY is released into the portal vessels at the time of the preovulatory LH surge (McDonald *et al.*,1987; Sutton *et al.*, 1988). However, NPY alone does not stimulate LH secretion from the anterior pituitary, but acts to potentiate the pituitary response to exogenous GnRH, *in vitro* and *in vivo* (Crowley *et al.*, 1987; Sutton *et al.*, 1988; Bauer-Dantoin *et al.*, 1991; O'Conner *et al.*, 1993). Pituitary responsiveness just prior to the LH surge has been ascribed to the priming actions of estradiol or to GnRH self priming actions (Aiyer and Fink, 1974). However, Bauer-Dantoin *et al.* (1991) demonstrated that NPY may also function to prime the pituitary before the preovulatory LH surge, as NPY administered concomitantly with pulsatile exogenous GnRH, greatly enhanced LH surges in pentobarbital anaesthetized proestrus rats.

The results from these studies, provide evidence for a physiological role for NPY in regulating the pulsatile and surge secretion of LH in the OVX and intact rat. There is also evidence to suggest that NPY mediates the actions of poor nutritional status on reproductive function. However, although NPY immunoreactive neurons and receptors have been identified in the forebrain of the pig (Busch-Sorensen *et al.*, 1989) there have been no definitive studies to determine whether NPY plays a physiological role in the regulation of GnRH secretion in this species.

2.5.15 Serotonin

Central serotonergic neurons arise mainly from the mid-brain raphe nuclei, with the dorsal and median raphe nuclei innervating the hypothalamus (reviewed by Weiner *et al.*, 1988). There is evidence that some serotonergic neurons may originate within the hypothalamus, as deafferentation of the hypothalamus does not completely deplete serotonin (5-HT) concentrations (Palkovits, 1979). 5-HT neurons and GnRH neurons have been identified in close apposition in the POA, ME and the OVLT (Jennes *et al.*, 1982), and synaptic contacts have been identified between the two systems in the MPOA (Kiss and Halasz, 1985) in the rat, providing an anatomical basis for the regulation of GnRH by 5-HT. In OVX rats there is a circadian rhythm of 5-HT metabolism in the MPOA, suprachiasmatic nucleus, anterior hypothalamus, MBH and ARC, but not the ME (Meyer and Quay, 1976; Kan *et al.*, 1977; Wirz-Justice, 1987). However, this rhythm is thought to have implications for the regulation of PRL secretion, and not LH secretion (Arey and Freeman, 1990). Both stimulatory and inhibitory effects on GnRH/LH secretion have been reported for 5-HT. Similar to other neurotransmitters, these effects are mediated by different receptor types and the prevailing steroidal milieu. It is believed that any effects on LH secretion by 5-HT are mediated at the level of the hypothalamus, as i.c.v. injection of 5-HT did not affect the pituitaries responsiveness to exogenous GnRH in proestrous rats (Morello *et al.*, 1989), but did decrease plasma LH concentrations, whereas systemic 5-HT had no effect on LH concentrations (Becu de Villalobos *et al.*, 1984).

Stimulation and lesioning studies of the raphe nucleus have been employed to determine the serotonergic contribution to LH secretion. Stimulation of the dorsal raphe nucleus led to decreased plasma LH concentrations in OVX rats; this effect could be blocked

by prior administration of metergoline, a 5-HT receptor antagonist, although metergoline alone had no effect on pulsatile LH secretion (Arendash and Gallo, 1978). Destruction of 5-HT neurons with p-chloroamphetamine also does not affect any of the characteristics of pulsatile LH secretion in the OVX rat (Rasmussen *et al.*, 1981). In a similar study, microinjection the 5-HT neurotoxin, 5,7-DHT, into discrete hypothalamic nuclei resulted in differential effects on LH secretion; administration into the MPOA resulted in decreased LH secretion, and administration into the MBH increased basal LH secretion (Johnson and Crowley, 1983). Furthermore, 5,7-DHT decreased mean LH secretion by decreasing pulse frequency, as amplitude was not affected. Fifty-five days after lesioning of 5-HT neurons, pulsatile LH had returned to normal control levels, indicating that 5-HT plays a facilitory role in the regulation of GnRH secretion and that other neurotransmitters regulate GnRH/LH secretion in place of 5-HT (Johnson and Crowley, 1983). Conversely, Petersen *et al.* (1989) found that while electro-stimulation of the dorsal raphe nuclei increased 5-HT concentrations in the MPOA and the paraventricular nuclei, but not in the ARC or ME, there was no effect on LH release in OVX estrogen-primed rats. These studies indicate that while 5-HT may inhibit LH secretion, it does not appear to have a physiological role in the continued pulsatile secretion of LH in the OVX rat.

The paradoxical influences of 5-HT on GnRH/LH secretion are mediated by different receptor types, and varying estrogen concentrations may activate these receptors independently, thereby mediating both the stimulatory and inhibitory actions of 5-HT on GnRH release (as reviewed by Vitale and Chiocchio, 1993). *In vitro* administration of 5-HT to MPOA and MBH tissue from OVX and OVX estradiol-primed rats resulted in decreased and increased release of GnRH, respectively (Meyer, 1989). Quipazine, a 5-HT_2 agonist, inhibits LH secretion in the OVX rat. This action can be reversed by the antagonists, ketanserin (5-HT_2), metergoline and methysergide (Lynch *et al.*, 1984; Johnson and Kitts, 1988). It has been demonstrated that 5-HT_1 receptors, which are considered to be mediating stimulatory actions, and 5-HT_2 receptors, which mediate inhibitory actions, are independently and differentially regulated by ovarian steroids (Lenahan *et al.*, 1986). Furthermore, it was suggested that 5-HT may mediate the estrogen negative feedback on LH secretion, as acute estradiol treatment in OVX rats suppressed LH secretion and decreased 5-HT metabolism in the MPOA, while increasing 5-HT turnover in the MBH (Johnston *et al.*, 1984). This study further supports a stimulatory role for 5-HT in the MPOA, at the level of the GnRH cell bodies, and an inhibitory influence in the MBH, at the level of the GnRH axons and terminals.

Johnson and Crowley (1983) also found that estradiol treatment decreased LH and increased 5-HT turnover; however, destruction of 5-HT terminals did not alter the suppressive effects of estrogen on LH secretion. Acute treatment (1 hour) of OVX rats with estrogen decreased the number of 5-HT_1 receptors, however 72-hour treatment with estrogen increased the number of 5-HT_1 receptors in estrogen concentrating areas of the hypothalamus, such as the POA/AH and MBH, ARC and ME (Biegon and McEwen, 1982). Meyer *et al.* (1992) demonstrated that *in vitro* blockade of 5-HT_2 receptors using ketanserin, or stimulation of 5-HT_1 receptors using the agonist, 8-OH-DPAT, significantly increased GnRH release from MPOA/MBH tissue from OVX estrogen-primed rats. Similarly, Héry *et al.* (1995) also found that 8-OH-DPAT increased *in vitro* secretion of GnRH from rat

hypothalamic cell cultures. These studies suggest that in an estrogenic environment, 5-HT_1 receptors mediate stimulatory effects, and 5-HT_2 receptors mediate inhibitory effects of 5-HT on GnRH secretion. In contrast to these studies, Tanaka *et al.* (1992) found that administration of ketanserin on the morning of proestrus in normally cycling rats, abolished the preovulatory LH surge and prevented ovulation, indicating an involvement of 5-HT in the surge generating mechanism via a 5-HT_2 receptor.

Morello *et al.* (1989) have suggested that the inhibitory actions of 5-HT may be mediated by GABA, as stimulation of the medial raphe nucleus at noon on proestrus, prevented the preovulatory LH surge and ovulation, and this effect was blocked by administration of methysergide or prior administration of the GABA antagonists, picrotoxin or bicuculline. Administration of 5-HT or GABA into the third ventricle at noon on proestrus also prevented the LH surge and ovulation. Pretreatment with methysergide prevented the effect of 5-HT but did not block the effect of GABA (Morello *et al.*, 1989 and 1991). This suggests that these two neurotransmitter systems may be linked in series, and that medial raphe nucleus stimulation or administration of 5-HT into the third ventricle stimulates GABA release, which in turn inhibits GnRH secretion.

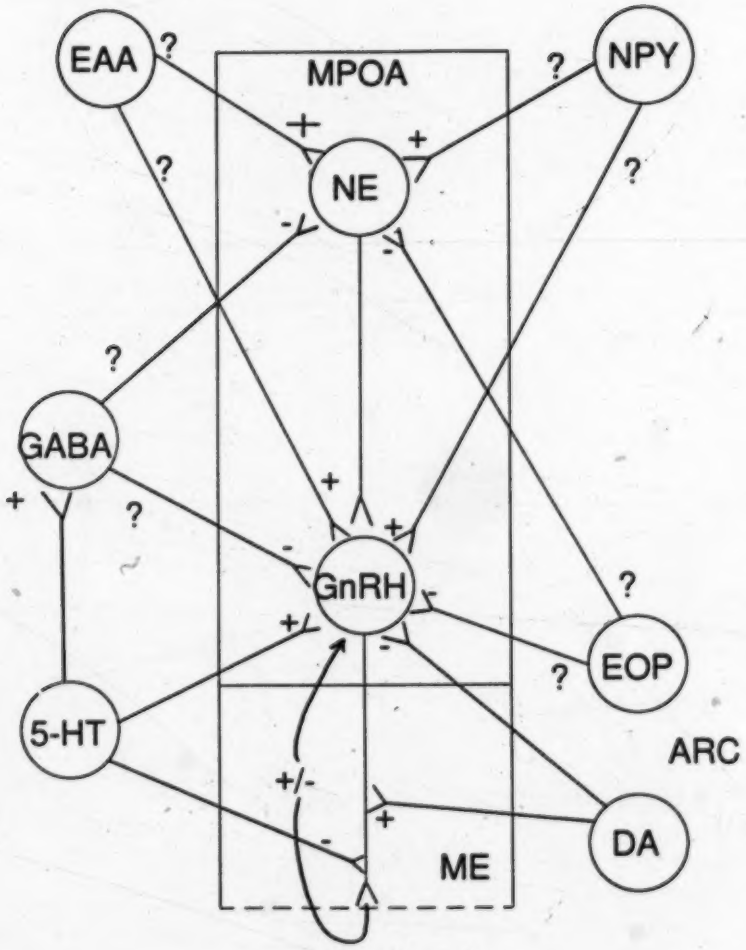
2.6 Conclusions

GnRH release, and the subsequent release of LH, are both key elements of reproduction in the mammal. Without central GnRH release, pulsatile LH release and subsequent follicular maturation and ovulation would not occur. The control of GnRH/LH is mediated by many factors, and it is quite clear that all of these inputs interact to modulate the GnRH pulse generator (Fig. 2.3). A huge body of work has already been completed to study the mechanisms involved in GnRH/LH secretion in laboratory species and primates. However, there are only a handful of recent studies on the central regulation of GnRH secretion and the effects on reproductive function available in the pig. There is still much work to be done to add to the sum of our knowledge on this complex system governing overall reproductive effectiveness in this species. The more that is known about the central regulation of GnRH secretion, the better we are able to manipulate the system and to improve many aspects of reproduction and fertility in the pig.

With these concepts and goals in mind, the following series of experiments were undertaken to further understand the regulation of GnRH secretion in the female pig in different physiological paradigms. As previously discussed, our lab has focussed on lactational anestrus in the sow, and the opioidergic mechanisms regulating this phenomena. Following from that, the experiment described in Chapter 3 was designed to determine whether there was an opioidergic system in place which was inhibitory to LH secretion immediately prior to parturition, and the period immediately post-partum, when there appears to be no functional opioidergic system in place (De Rensis *et al.*, 1993a). Chapter 4 describes the *in vivo* experiment designed to determine whether the EOP inhibit GnRH secretion at a pre-synaptic level, via inhibition of stimulatory noradrenergic neurons, or at a post-synaptically, directly at the level of the GnRH neuron. Due to difficulties experienced using the *in vivo* model to investigate the noradrenergic and opioidergic interactions regulating

GnRH secretion, an *in vitro* model was developed and is discussed in Chapters 5 and 6, and Appendix 1 of this thesis.

FIGURE 2.3 Diagrammatic representation of the possible neuroendocrine inputs regulating GnRH secretion, as discussed in this literature review. 5-HT = serotonin; ARC = arcuate nucleus; DA = dopamine; EAA = excitatory amino acid; EOP = endogenous opioid peptide; GABA = gamma-aminobutyric acid; GnRH = gonadotropin releasing hormone; ME = median eminence; MPOA = medial preoptic area; NE = norepinephrine; NPY = neuropeptide Y.


EAA
MPOA
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5-HT
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CHAPTER 3
Opioidergic Control of Luteinizing Hormone and Prolactin Secretion in Late Gestation in the Sow[1]

INTRODUCTION

In the sow, there may be two opioidergic mechanisms modulating luteinizing hormone (LH) secretion. One is progesterone dependent, as in the luteal phase, and the other is dependent on the suckling stimulus during lactation. Several studies have demonstrated that the steroid milieu of the animal is particularly important in the opioidergic modulation of LH (Cosgrove *et al.*, 1993 for review). In the cyclic gilt (Barb *et al.*, 1986a), the opioid antagonist naloxone was found to increase LH secretion only in the luteal phase of the cycle, when the concentration of plasma progesterone is high. However, during established lactation in the sow, when steroid concentrations are basal, the endogenous opioid peptides (EOPs) also appear to be the main inhibitors of LH (Barb *et al.*, 1986b; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a and 1988b; De Rensis, 1993; De Rensis *et al.*, 1993a).

During gestation in the sow, both LH and prolactin (PRL) are reported to have important luteotropic effects (Parvizi *et al.*, 1976; Ziecik *et al.*, 1983; Kraeling *et al.*, 1992; Szafranska and Tilton, 1993), and PRL also has important mammogenic and lactogenic actions (as reviewed by Rillema *et al.*, 1988). Studies in the rat (Gintzler, 1980; Wardlaw and Frantz, 1983; Dondi *et al.*, 1991; Sagrillo and Voogt, 1991; Sumner *et al.*, 1992; Zhen and Gallo, 1992), cow (Aurich *et al.*, 1990) and sow (Aurich *et al.*, 1993) have shown that opioidergic tone increases throughout gestation and modulates several endocrine events including LH secretion. Devorshak-Harvey *et al.* (1987) reported that in the rat, LH secretion was inhibited by the EOP during gestation. In one study using first parity sows, Szafranska *et al.* (1994) determined that LH secretion was inhibited by the EOP in mid-gestation but not later in gestation.

Generally it is believed that the main site of opioidergic inhibition of LH is hypothalamic, via inhibition of GnRH and noradrenergic neurons (Dyer and Grossmann, 1988; Rasmussen *et al.*, 1988; Clough *et al.*, 1990; Nishihara *et al.*, 1991; Chang *et al.*, 1993; Barb *et al.*, 1994). Immunohistochemical studies in the rat (Chen *et al.*, 1989; Horvath *et al.*, 1992), ewe (Conover *et al.*, 1993; Lehman and Karsch, 1993) and pig (Kineman *et al.*, 1988 and 1989; Leshin *et al.*, 1989) have demonstrated a close apposition of opioidergic fibres to GnRH and noradrenergic fibres, confirming that these interactions are possible. A recent study in the pig demonstrated that naloxone increased GnRH secretion from hypothalamic-preoptic explants and that this response was attenuated by coadministration of morphine (Barb *et al.*, 1994). However, there is evidence in the rat (Cacicedo and Sanchez-Franco, 1986; Blank *et al.*, 1986), cow (Chao *et al.*, 1986) and pig (Barb *et al.*, 1990) that opioidergic

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inhibition can occur at the pituitary level. In contrast to their effects on LH, the endogenous opioids are thought to be indirectly stimulatory to PRL, either via their inhibition of dopamine, the major putative PRL inhibiting factor, or alternatively, via stimulation of a PRL-releasing factor (Miki *et al.*, 1981; Knight *et al.*, 1986; Baumann and Rabii, 1991; Johnson *et al.*, 1991; Soaje and Deis, 1994). Consistent with these opposing effects, an inverse relationship has been observed between the pattern of LH and PRL secretion during lactation in the rat (Smith, 1978; Sirinathsinghji and Martini, 1984), human (Kremer *et al.*, 1991) and sow (Bevers *et al.*, 1983a and 1983b; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a; De Rensis, 1993; De Rensis *et al.*, 1993a).

In previous studies, focusing on the neuroendocrine mechanisms regulating LH and PRL secretion during lactation, our laboratory has used both naloxone (De Rensis, 1993; De Rensis *et al.*, 1993a) and morphine (De Rensis, 1993) administration to demonstrate an opioidergic response in established lactation. However, in very early lactation, the initial suckling-induced inhibition of LH appears to be independent of an opioidergic mechanism until approximately 54-72 hours after parturition. The results of Szafranska *et al.* (1994) suggest that the absence of opioidergic regulation of LH in early lactation might be a carry over effect from late pregnancy, although these observations appeared to contradict the general concept that active opioidergic regulation of LH secretion exists in a high steroidal milieu. Alternatively, the neuroendocrine events of the peri-parturient period may lead to a temporary abrogation of the opioidergic inhibitory inputs to GnRH and LH secretion. The correct interpretation of our work on the neuroendocrine regulation of LH and PRL secretion in the immediate post-partum period depends on a clear understanding of the status of the opioidergic system in late gestation. Therefore, the primary goal of this study was to determine whether or not the EOPs modulate LH and PRL secretion just prior to farrowing, when plasma progesterone concentrations are still high. We also wished to use this opportunity to further explore a luteotropic role for LH and PRL in gestation suggested in earlier studies.

MATERIALS AND METHODS

Animals and Blood Sampling

Ten Camborough sows of mixed parity from the University of Alberta Swine Research Unit were surgically implanted with an indwelling jugular vein catheter via the cephalic vein on d105.3 ± 1.57 (standard deviation (S.D.)) of gestation, two days prior to the beginning of the experiment. At this time sows were moved from gestation room stalls into farrowing crates. Average (± S.D.) weight of the sows during the experiment was 221.4 ± 49.1 kg. A lighting regimen of 12L:12D (lights on at 0600h) was in place for the duration of sampling. Water was available *ad libitum* and sows were fed a standard commercial dry sow diet at National Research Council recommended gestation allowances based on their metabolic body weight.

During two periods of 12 h (0600-1800h) on two consecutive days between 105 and 110 of gestation (means (± S.D.) = d107.7 and d108.7 ± 1.49), 3-ml blood samples were taken at 10 min intervals. As a repeat measures design, an equal number of sows received 2.0 mg/kg of the opioid antagonist, naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO;

50 mg/ml in sterile saline) 6 hours after sampling began via the cephalic vein catheter, followed by two further 1.0 mg/kg injections at hourly intervals, on the first or the second day of sampling (treatment days); these sows acted as untreated controls on the alternate day (control days) (Fig.3.1). This pattern and dosage of naloxone has been used previously to elicit effective opioidergic antagonism (De Rensis, 1993). Samples were collected into heparinized tubes and centrifuged at 1,500 x g at 4°C for 15 min and the plasma was frozen at -30°C until assay for LH, PRL and progesterone. Catheters were flushed with 2-ml heparinized saline (10 IU/ml) after each blood sample. At the end of the second sampling day, the catheters were removed; the sows farrowed normally (d114.5 ± 1.69 (S.D.)) and were returned to the herd after weaning.

Hormone Assays

Plasma LH, PRL and progesterone were quantified in all samples by RIA. Plasma LH concentrations were determined in 200 µl aliquots of plasma using the double-antibody RIA described by De Rensis *et al.* (1993b). The purified porcine LH used for iodination and standards was kindly supplied by Dr. J.H.F. Erkens (Research Institute for Animal Production, Ziest, Netherlands) and Dr. S.D. Glenn (Alton Jones Cell Science Center, Lake Placid, NY, USA), respectively. The intra- and interassay coefficients of variation (CV) were 7.8% and 11.5%, respectively. The sensitivity of the assay, defined as 80% of total binding, was 0.014 ng/tube. Plasma concentrations of PRL were routinely measured in 50 µl of plasma by the method described by de Passillé *et al.* (1993), using purified porcine PRL (USDA-pPRL-B-1) for iodination and standards generously provided by Dr. S. Raiti (USDA Animal Hormone Program and the National Hormone and Pituitary Program, Beltsville, Maryland). Intra- and interassay CVs were 9.1% and 12.3%, respectively. Sensitivity of the assay, defined as 76% of total binding, was 0.22 ng/tube. Plasma progesterone was routinely extracted from 100 µl of plasma and assayed by the method described by Pharazyn *et al.* (1991), with the use of progesterone antibody (rabbit A-18) kindly provided by Dr. N.C. Rawlings (Department of Veterinary and Physiological Science, Western College of Veterinary Medicine, University of Saskatchewan). The average assay extraction efficiency was 69.4% and estimated potencies were corrected for recovery. Assay sensitivity, defined as 84% of total binding, was 0.03 ng/tube. The intra- and inter-assay CVs were 5.7% and 18.5%, respectively.

Statistical Analysis

Plasma LH and PRL data were initially characterized by the method of Shaw and Foxcroft (1985) through use of a sliding window technique, and mean LH and PRL concentrations, over defined periods, were used for statistical analysis. LH pulsatility was visually appraised according to the criteria established by Cosgrove *et al.* (1991). Data for each 12-h sampling day were split into two 6-h periods, designated as Periods 1 and 2 on control days and Periods 3 and 4 on days of naloxone treatment. ANOVA for repeated measures (PROC GLM, SAS statistical package) was applied to mean LH and PRL data to assess naloxone effects both across (Periods 2 and 4) and within (Periods 3 and 4) days; "order" (order of treatment; i.e., naloxone on Day 1 or naloxone on Day 2) was initially fitted

as a main effect. Maximum and minimum values for LH data were also analysed with this statistical model. The relationship between LH and PRL secretion was examined using regression analysis (PROC REG) on mean hormone concentrations. The daily pattern of PRL secretion was examined using a polynomial regression model for individual sow profiles and on mean PRL profiles for control and naloxone days. The absolute nadir of the mean PRL profiles for both days was considered to be the point at which the slope of the polynomial regression was equal to zero.

Progesterone and LH profiles for individual sows were compared through use of an experimental time-lag analysis program provided by R.Weingard (personal communication). This program is similar to a time series analysis program that identifies relationships between two data sets, progesterone and LH in this case, at different time lags. Progesterone profile data remained stationary and LH profile data were lagged to the right, based on the principle that LH drives progesterone production by the CL, and each lag shift represented a 10 minute interval. The program then compared the remaining profile points, determined a best fit correlation coefficient, and tested these for statistical significance using a correlation coefficient test. Significant positive or negative correlations indicate that changes in LH secretion correlate to changes in progesterone secretion a certain time later. A t-test comparison of control day vs naloxone day lag times was made and respective mean lag times were calculated.

RESULTS

"Order" (order of treatment, i.e., naloxone on Day 1 or Day 2) had no significant effect on LH (P=0.57) or PRL (P=0.94).

Plasma LH

There was a significant (P<0.03) period x day interaction for mean plasma LH concentrations. Therefore, further analysis of naloxone effects both within day and across days were conducted. Opioid antagonism with naloxone in late gestation elevated mean LH concentrations whether the comparison was made within days (period 4 vs 3: P=0.007) or across days (period 4 vs 2: P=0.003) (Fig.3.2). A composite profile of the 10 sows (Fig.3.3a) clearly demonstrates the effect of naloxone administration on LH secretion. An individual sow profile (Fig.3.3b) shows low LH pulsatility in the control periods, approximately 1 pulse every 4 hours, and increased LH pulsatility after naloxone injection, indicating that EOPs were inhibiting LH secretion. Maximum and minimum LH secretion were also significantly increased by naloxone administration (Table 3.1), although there was no overall significant effect on pulsatility.

Plasma Prolactin

There was a significant (P<0.015) period x day interaction for mean plasma PRL concentrations. Therefore, as with LH, further analysis of naloxone effects both within day and across days was conducted. Opioid antagonism with naloxone in late gestation suppressed mean PRL concentrations when the comparison was made within (Period 4 vs 3: P=0.0067) but not across days (Period 4 vs 2: P=0.098) (Fig.3.4). To determine if the

apparent decline in PRL secretion in response to naloxone might be due to an endogenous daily rhythm rather than to treatment, mean PRL concentration in Periods 1 and 2 on the control day were compared but they were not different (P=0.66). Reference to individual patterns of PRL secretion (Fig.3.5) confirms the decrease in prolactin secretion after naloxone treatment. Consideration of the overall responses to treatment suggest an inverse pattern of secretion between LH and PRL in response to naloxone. However, regression analysis showed that LH and PRL are significantly positively correlated throughout the control day (Periods 1 and 2; P<0.05) (Fig.3.6a) and the within-day control period (Period 3; P<0.005) (Fig.3.6b), after treatment with naloxone, this correlation was abolished (P>0.10) (Fig.3.6c).

Polynomial regression analysis of individual sows confirmed a daily rhythm in PRL secretion on control days in 7 of 10 sows ($P \leq 0.04$). Naloxone abolished or altered this rhythm in 4 of these 7 sows ($P \geq 0.13$). When composite profiles of the 10 sows were analysed, significant (P<0.0001) regressions were established on both control and naloxone days. However, comparison of the mean PRL profiles (Fig.3.7) suggests that the expected afternoon increase in PRL secretion is blocked by naloxone; the absolute nadir in PRL concentrations preceding the afternoon increase occurred at 1240 h on control days but was delayed until 1630 h on naloxone days.

Plasma Progesterone and LH

Mean progesterone concentrations did not differ significantly between periods (P=0.91) and was 24.96 ± 0.50 (± SEM) ng/ml over both days. Dependent on the time lag fitted in the model, both significant ($P \leq 0.05$) positive and negative correlations between LH and progesterone concentrations were established in 9 of the 10 sows on control days and in all of the sows on naloxone treatment days and are presented in Table 3.2. The estimated mean lag time between an LH episode and a possible progesterone secretory response (based on positive correlations) was 73.75 ± 17.92 vs 28.33 ± 15.58 minutes for control vs naloxone days, respectively, with a trend (p=0.09) for progesterone to respond faster to LH stimulation on naloxone days than control days. Figure 3.8 (a, b and c) illustrates the individual variance between sows and the difficulty of establishing a consistent functional relationship between LH and progesterone secretory events.

DISCUSSION

We believe this is the first substantive study investigating the opioidergic regulation of LH and PRL secretion in what is truly late gestation in the sow. A better understanding of the switch from a steroid-dependent mechanism regulating LH and PRL secretion during pregnancy to a steroid-independent mechanism during established lactation in the sow would contribute considerably to an appreciation of hypothalamic-pituitary control in changing reproductive states, especially as this transition appears to involve a period immediately after parturition when EOP regulation is ineffective. An important objective of the present study was therefore to clearly establish the status of opioidergic regulatory inputs to LH and PRL secretion in very late pregnancy. Naloxone administration antagonized the EOP and significantly increased mean, maximum and minimum plasma LH, and decreased mean plasma

PRL concentrations, within one week of parturition. Studies in other species, as well as in the pig, have shown that opioidergic tone increases during gestation and that EOPs regulate LH and PRL secretion. Hypothalamic ß-endorphin levels and the concentration of μ-opioid receptors have been shown to increase in the later half of pregnancy in rats (Dondi *et al.* 1991) and cattle (Aurich *et al.*, 1990) and plasma ß-endorphin concentrations were found to be higher in sows during late pregnancy, than in non-pregnant sows (Aurich *et al.* 1993). Plasma PRL significantly increased in ewes during late gestation in response to intrafetal morphine injection and decreased in response to intrafetal naloxone injection, indicating a role of opioids in regulating PRL secretion in the ewe in late gestation (McMillen and Deayton, 1989). Naloxone was able to prevent the nocturnal surge of PRL that occurs during the first half of pregnancy in rats, indicating an EOP involvement in maintenance of pregnancy in this species. It was further demonstrated that the EOPs stimulated PRL secretion by inhibition of tuberoinfundibular dopaminergic neurons (Sagrillo and Voogt, 1991). Kappa opioid receptors in the medial preoptic area and mediobasal hypothalamus are involved in EOP suppression of LH secretion during mid-pregnancy in the rat (Zhen and Gallo, 1992). Although Rund *et al.* (1990) were unable to demonstrate an LH response to naloxone treatment in late gestation in beef cows, recent work with pregnant gilts has suggested that the EOP modulate LH at day 40, but not at day 70 of gestation (Szafranska *et al.*, 1994). It is likely that the differences in these findings are attributable to the number of animals and the sampling periods used, as Szafranska *et al.* (1994) reported that although individual sows in the late pregnancy group responded positively to naloxone, the overall effect of treatment was not significant.

The results of the present study are consistent with data from cyclic or ovariectomized animals suggesting that the inhibitory effects of the EOP are steroid dependent (as reviewed by Kalra *et al.*, 1988; Haynes *et al.*, 1989; Cosgrove *et al.*, 1993). These reviews indicated that the EOP could also be mediating steroid negative feedback effects on LH secretion. Previously, Barb *et al.* (1985, 1986a and 1988) demonstrated that naloxone was able to increase LH secretion in the luteal phase and in ovariectomized, progesterone treated gilts, but not in the early follicular phase or in prepubertal gilts. Conversely, effective but steroid-independent, EOP suppression of gonadotropin secretion appears to exist in lactation in the pig. In a series of similar studies Barb *et al.* (1986b), Mattioli *et al.* (1986) and Armstrong *et al.* (1988a) found that naloxone increased episodic LH release but decreased peripheral PRL during established lactation in the sow. However, although De Rensis *et al.* (1993a) showed that a single injection of naloxone on d10 of lactation (the positive control for this experiment) caused a significant increase in LH secretion and decrease in PRL, naloxone failed to reverse suckling induced effects on LH and PRL secretion before 78 hours postpartum. Available evidence therefore supports a role for endogenous opioids in the regulation of LH and PRL during mid and late lactation, but not in the early post partum period. Use of the opioid agonist, morphine, during lactation confirms the naloxone results in that morphine suppressed LH secretion in established lactation (Armstrong *et al.*, 1988b; De Rensis, 1993); however, unexpectedly it also suppressed PRL secretion. This effect may have been due to the suckling stimulus making PRL release refractory to morphine stimulus (Callahan *et al.*, 1988). Together these studies strongly suggest that EOP's are closely

associated with the inhibition of LH and stimulation of PRL secretion during lactation.

In the present study, mean (± SEM) LH for control Periods 1-3 was 0.28 ± 0.02, 0.29 ± 0.02, 0.30 ± 0.03 ng/ml, respectively. As previously reported (Parvizi *et al.*, 1976; Kraeling *et al.* 1992; Ziecik *et al.* 1982/1983), LH was secreted in an episodic pattern in late gestation, and the frequency of LH episodes of approximately 1 pulse every 4 hours was similar to that observed by Smith and Almond (1991). Ziecik *et al.* (1983) stated that LH peaks were less numerous and of lower amplitude in the second half of pregnancy, and Parvizi *et al.* (1976) reported that serum LH concentrations between days 90 and 94 of gestation were not different from values immediately before parturition. Kraeling *et al.* (1992) observed a declining LH pulse frequency in pregnant gilts between days 30 and 110, and episode frequency at d110 was comparable to that seen in the present study. Although there was a measurable increase in mean, maximum, and minimum LH concentration in response to naloxone (Table 3.1), we were unable to establish a significant effect on episodic secretion. It is likely that this was due to the low episodic frequency at this stage of gestation and therefore an inability to characterize changes in frequency over a 6-h period, as necessitated by the acute treatment protocol. As can be seen in the individual sow profile (Fig. 3.2b), there is an increase of one episode in 6 h when the last 6 h of each day are compared. This marginal increase is not statistically measurable; however, there is a clear effect of continuously elevated baseline (Fig. 3.2b; Table 3.1, LH minimum). When frequency reaches approximately 1 pulse per hour, as seen in the naloxone-treated period, such an increase in baseline would be expected. Together, these studies indicate that throughout gestation although mean LH may not differ, pulse frequency declines significantly as gestation progresses. At d110, pulse frequency is very similar to that of luteal phase LH secretion (Ziecik *et al.*, 1982/1983) when progesterone is also elevated and the EOP appear to play a major inhibitory role.

During control periods, mean PRL concentrations in our study did not differ markedly from those measured between days 34-36, prior to treatment with bromocriptine, in first parity gilts (Szafranska and Ziecik, 1990) or in the control gilts in the study by Szafranska and Tilton (1993). Interestingly, a daily rhythm of PRL secretion was identified in late gestation, and we are not aware of any previous reports of a daily rhythm of PRL secretion in the pig. In humans it is known that PRL secretion is pulsatile and PRL concentrations increase during periods of sleep (Tennekoon and Lenton, 1993). In the rat, PRL secretion remains constant and is secreted in a pulsatile manner throughout the estrous cycle with the exception of the proestrus surge (Lafuente *et al.*, 1993) and two daily surges in response to mating that continue until d10 of pregnancy (Sagrillo and Voogt, 1991). In the female Djungarian hamster PRL secretion in late gestation shows a pattern remarkably similar to that of the gestating sow seen in the current study. In hamsters, maximum PRL concentrations are seen nocturnally, decrease during the morning to reach a nadir by approximately mid-day, and then rise again in the late afternoon (Edwards *et al.*, 1995).

In the present study, LH and PRL were found to be positively correlated during the entire control day and the control period of the treated day, but after naloxone administration this relationship was abolished. Barb *et al.* (1986a) demonstrated that PRL secretion is under different opioidergic influences at varying stages of the estrous cycle and that there is a

synchrony between LH and PRL secretion during the luteal phase. However, in contrast to what was observed in the present study, naloxone was able to increase mean concentrations of both hormones. Similarly, Tennekoon and Lenton (1993) found a significant correlation between LH and PRL secretion during the mid luteal phase in a group of regularly cyclic women. The late gestation sow model also differs from the lactating sow model in which LH and PRL are inversely related and suggests that progesterone modifies opioidergic modulation of LH and PRL secretion in differing reproductive states.

The mean (± SEM) plasma concentration of progesterone in this study, 24.96 ± 0.5 ng/ml, is similar to that reported by Parvizi *et al.* (1976) but higher than other reports for sows at this stage of gestation. Beginning at approximately two weeks prior to parturition, Baldwin and Stabenfeldt (1975) characterized progesterone secretion in the sow and observed that plasma concentrations decreased from around 15 ng/ml at 100 days of gestation, to 10 ng/ml at d111 (2 days prior to parturition), and then to 3-4 ng/ml at parturition. In the gilt, progesterone concentrations decreased from 22.1 ± 0.4 ng/ml during early gestation (d39-44) to 18.2 ± 0.4 ng/ml during mid-gestation (d69-74) (Smith and Almond, 1991). Progesterone in gilts was reported as 8.9 ± 0.3 ng/ml at d34-36 and 14.6 ± 0.7 ng/ml at d43-45 after bromocriptine treatment (Szafranska and Ziecik, 1990). Szafranska and Tilton (1993) report that in their control gilts plasma progesterone concentrations between d60-66 were 12.6 ± 0.2 ng/ml and in their hyperprolactinemic group 20.8 ± 0.6 ng/ml. The discrepancy between progesterone concentrations in various studies could be due to differences in assay or extraction methods for this hormone.

The method used to identify a relationship between progesterone and LH secretion in this study was similar to that used in Tennekoon and Lenton (1993) where they identified a synchronous secretion of LH and PRL during the normal menstrual cycle. In this study, the absence or presence of positive correlations between hormone profiles is not an absolute indicator of a relationship between progesterone and LH secretion. Many strong negative correlations were also identified indicating that the profiles are out of phase, as LH increases, progesterone at some point is decreasing. A clearer relationship between LH and progesterone would be difficult to demonstrate as frequent peripheral measurements of progesterone show great fluctuation and therefore may mask any clear effect of LH episodes. Although an increase in progesterone secretion might have been expected in response to naloxone, given that LH secretion increased and LH is acting in a luteotropic manner, no such response was seen. This lack of an effect of naloxone could simply be masked by variability in peripheral progesterone *in vivo*.

A lag time of 0 minutes, suggested by the data in Figure 3.7a, does not seem physiologically possible. In all likelihood it represents an LH episode (LH1) occurring earlier than sampling was initiated and it is simply chance that the next LH episode (LH2) coincides exactly with the increase in progesterone secretion seen in response to the unmeasured LH1. It would appear that LH episode frequency is the final determinant of progesterone lag time relationships, therefore time lags may differ between animals having different LH frequency intervals. On control days, sows with similar LH pulse frequencies, approximately 2 hours between LH episodes, had similar lag delays before the CL responded, between one and two hours later, with increased progesterone secretion. Parvizi *et al.* (1976) originally identified

a similar relationship between LH and progesterone occurring 3 weeks prior to parturition in Göttingen mini sows in which 3 out of 4 increases in LH were followed by a rise in progesterone at approximately 40 to 60 min intervals. By 41-17 hours prior to parturition this relationship was greatly diminished and the time between episodes of LH and progesterone increased to approximately 120 minutes. A similar steroid response to LH occurs in rams (Sanford *et al.*, 1974; Falvo *et al.* 1975), in which an increase in LH secretion results in increased testosterone production. In the anestrus (Scaramuzzi and Baird, 1977) and the luteal phase (Baird *et al.* 1976) ewe, LH pulses result in a rise of estradiol 25 minutes and 30 minutes later. However in the luteal phase ewe (Baird *et al.*, 1976) progesterone secretion was not correlated to LH secretion.

Although literature suggests that the corpus luteum of the non-pregnant sow is autonomous (see Foxcroft and Van De Wiel, 1982 for review), a number of studies suggest a luteotropic role for LH in the maintenance of the corpora lutea of pregnancy (Ziecik *et al.*, 1982/1983; Wiesak 1985; Szafranska and Ziecik, 1990; Szafranska *et al.*, 1992). Data supporting a luteotropic role for PRL in maintenance of pregnancy in the sow are more equivocal. The number of PRL binding sites on the CL increase during the first half of gestation (Rolland *et al.* 1976) with the greatest increase occurring by d60 (Jammes *et al.*, 1985), providing a mechanism by which PRL could fulfil a luteotropic role. However, Cook *et al.* (1967) reported that although LH was able to stimulate progesterone secretion *in vitro* from luteal tissue obtained during the first half of pregnancy, PRL and FSH had no effect on steroid synthesis. Similarly, Wiesak (1985) did not find any effect of PRL on progesterone secretion from luteal cells taken on d18 or d19 of gestation. In contrast, large luteal cells are reported to secrete progesterone in response to PRL (Gregoraszczuk, 1990) and PRL stimulated progesterone synthesis from luteal tissue taken from gilts at d80 of gestation, in which LH had previously been immunoneutralized (Szafranska *et al.*, 1992). Although the low animal numbers used in the study of Taverne *et al.* (1982) probably precluded statistically significant results, their data suggested that the administration of the dopaminergic agonist, bromocriptine, *in vivo* reduced PRL in pregnant sows and caused early luteolysis and parturition. In contrast, Szafranska and Ziecik (1990) reported that although bromocriptine administration *in vivo* between days 37-42 of pregnancy decreased PRL secretion, this did not affect normal pregnancy, indicating that PRL had no essential role in luteal function at this time. Furthermore, Szafranska and Tilton (1993) made gilts hyperprolactinemic by using haloperidol from d60-66 of gestation; although this treatment suppressed LH secretion, progesterone concentrations were increased as compared with control values and abortion did not occur in any of the animals. Taken together, these results indicate that in early pregnancy in the sow LH appears to be the predominant luteotropin, responsible for progesterone production and pregnancy maintenance up to d50 of gestation. However, during the second half of pregnancy PRL also exerts luteotropic actions and likely acts synergistically with LH in luteal maintenance until parturition. Study of the co-ordinated regulation of LH and PRL in late pregnancy is therefore of physiological significance. The establishment of significant positive or negative correlations between LH and progesterone in the present study, and the demonstrated associations between LH and PRL, discussed above, are entirely consistent with the concept that LH and PRL are components of the

luteotropic complex in late gestation in the sow.

Therefore, with respect to the primary objective of this experiment, the results indicate that 1) the EOP inhibit LH secretion as late as d108 of gestation in the sow; 2) LH and PRL secretion are positively correlated in late gestation but opioidergic antagonism disrupts this relationship; 3) a daily rhythm of PRL secretion exists that is also disrupted by opioidergic antagonism; and 4) despite the difficulties of critically analysing results from unmodified sows *in vivo*, our data are consistent with the concept that LH and PRL are functionally related in a luteotropic complex during late gestation in the sow.

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FIGURE 3.1 Schematic diagram of the experimental design showing a representative treatment regimen for a sow. During two periods of 12 h (0600-1800h) on two consecutive days, d107 and d108, of gestation, 3-ml blood samples were taken at 10 min intervals. As a repeat measures design, an equal number of sows received 2.0 mg/kg of the opioid antagonist, naloxone, 6 hours after sampling began via the cephalic vein catheter, followed by two further 1.0 mg/kg injections at hourly intervals, on the first or the second day of sampling (treatment days); these sows acted as untreated controls on the alternate day (control days).

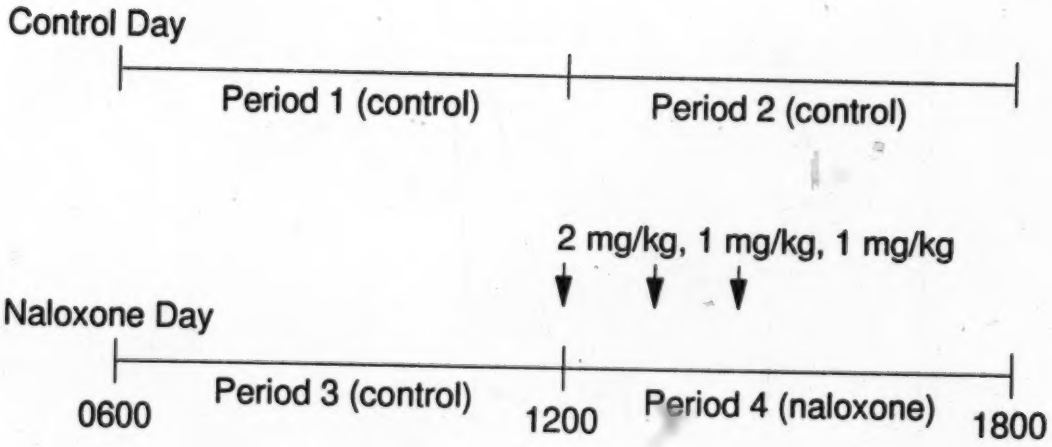

Control Day
Period 1 (control)
Period 2 (control)
2 mg/kg, 1 mg/kg, 1 mg/kg
Naloxone Day
0600
Period 3 (control)
1200
Period 4 (naloxone)
1800

FIGURE 3.2 Mean (+ SEM) plasma LH concentrations on d107.7 and d108.7 ± 1.49 (mean ± S.D.) of gestation in a group of 10 sows. Periods 1 and 2 represent across day controls, Period 3 is a within-day control and Period 4 is the naloxone treatment period. Means with different superscripts differ ($P<0.007$); ^{a,b} comparisons within day, ^{c,d} comparisons across days.

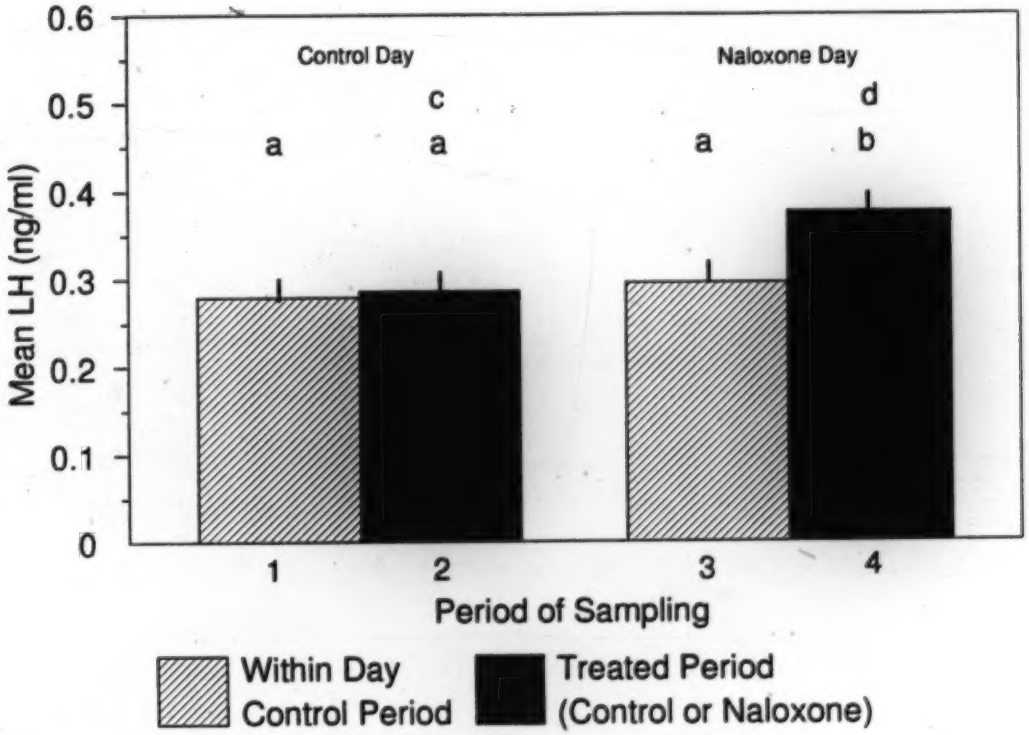
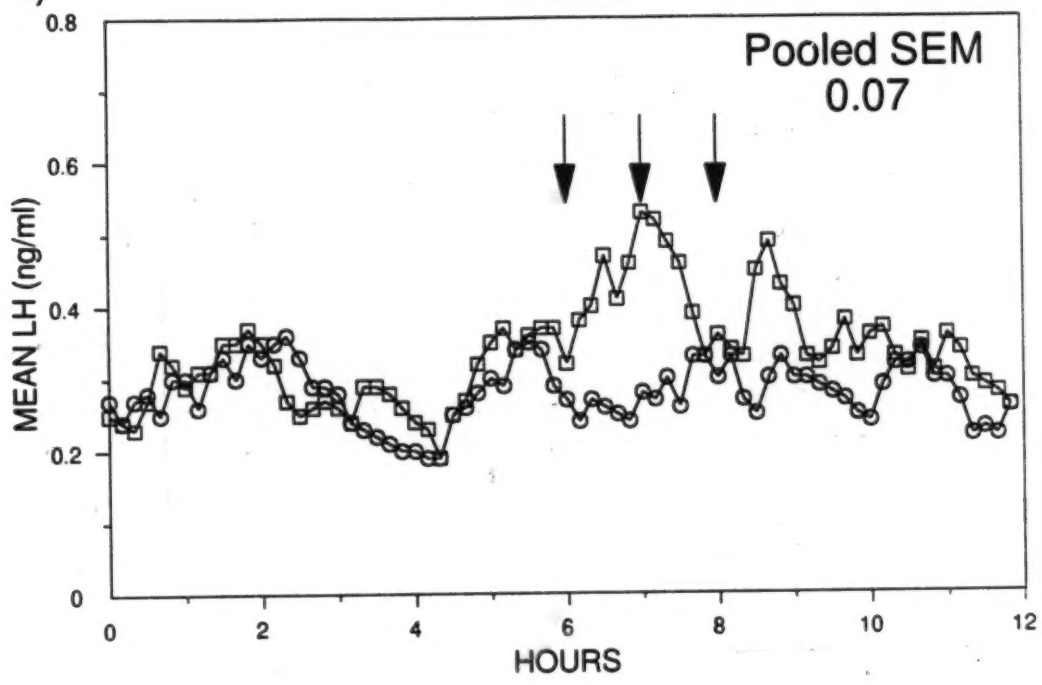

0.6
0.5
0.4
0.3
0.2
0.1
0
Mean LH (ng/ml)
Control Day
Naloxone Day
a
c
a
d
a
b
1
2
3
4
Period of Sampling
Within Day
Control Period
Treated Period
(Control or Naloxone)

FIGURE 3.3 a) Mean plasma LH profiles for 10 sows on control (circles) and naloxone (squares) days. b) Plasma LH profiles for the control and naloxone day for an individual sow. Arrows indicate naloxone injections (2 mg/kg, 1 mg/kg, 1 mg/kg, respectively). Solid circles indicate values that were below the sensitivity of the assay.

a)



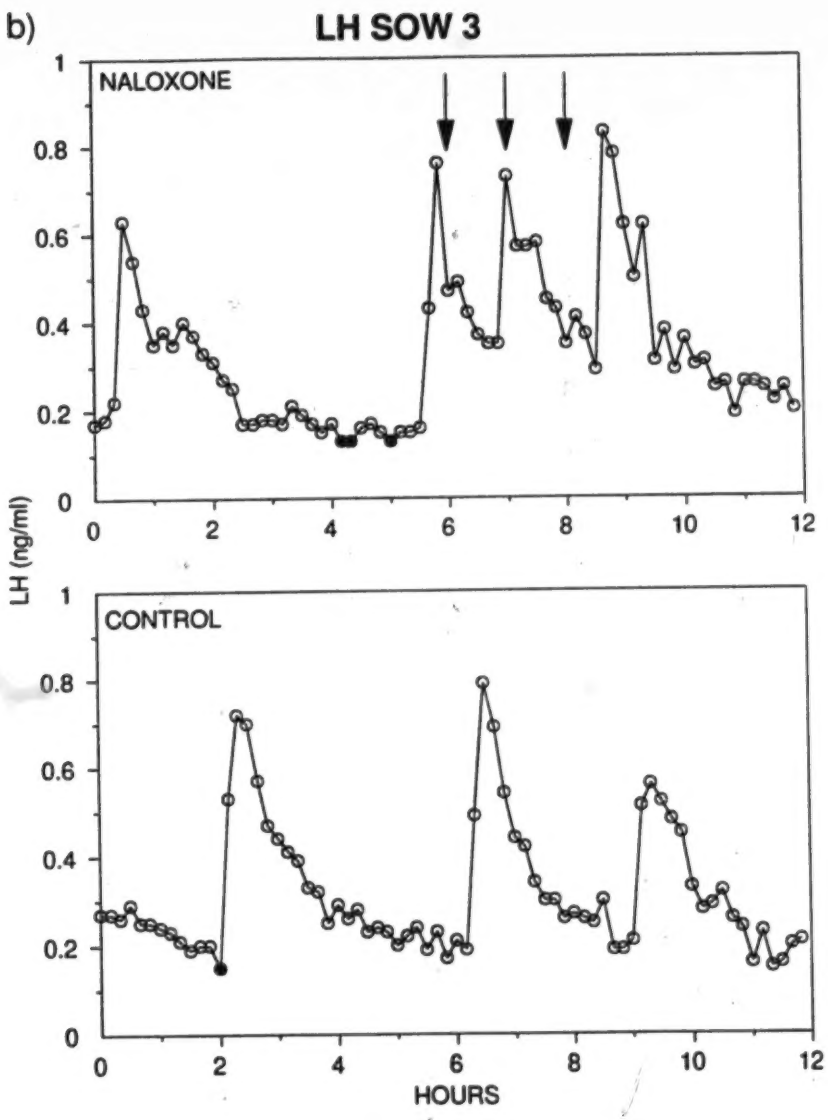

b)
LH SOW 3
NALOXONE
CONTROL
LH (ng/ml)
HOURS
0
0.2
0.4
0.6
0.8
1
0
2
4
6
8
10
12

FIGURE 3.4 Mean (+ SEM) plasma PRL concentrations on d107.7 and d108.7 ± 1.49 (mean ± S.D.) of gestation in a group of 10 sows. Periods 1 and 2 represent across day controls, Period 3 is a within day control and Period 4 is the naloxone treatment period. Means with different superscripts differ ($P<0.006$); ^{a,b} comparisons within day, ^{c,d} comparisons across day.

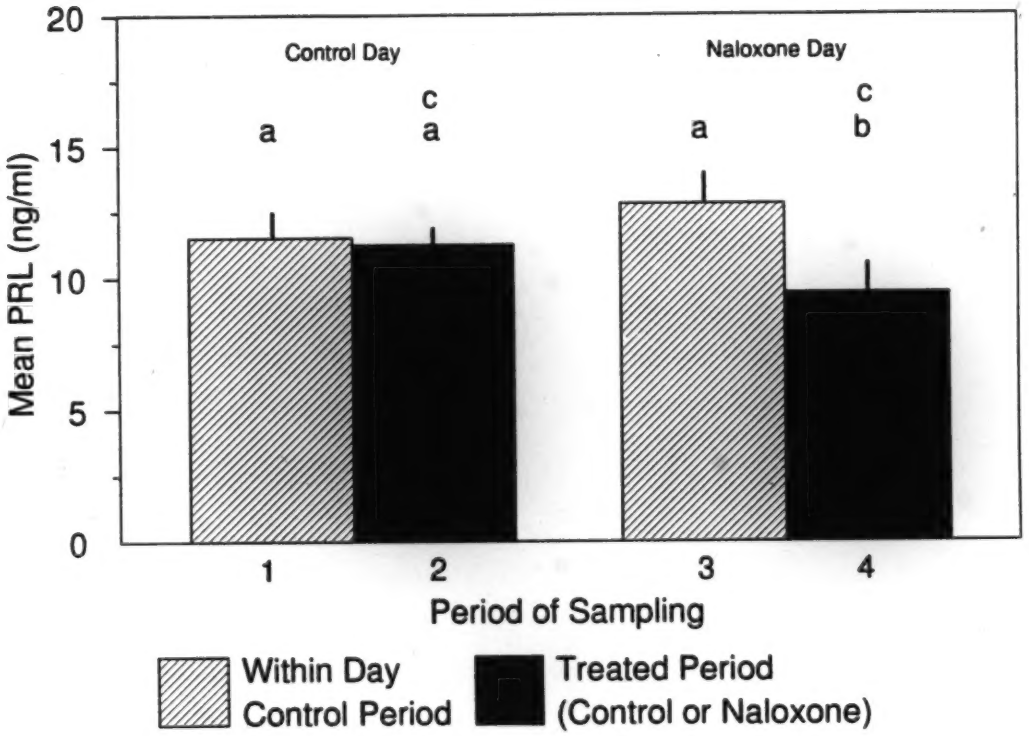
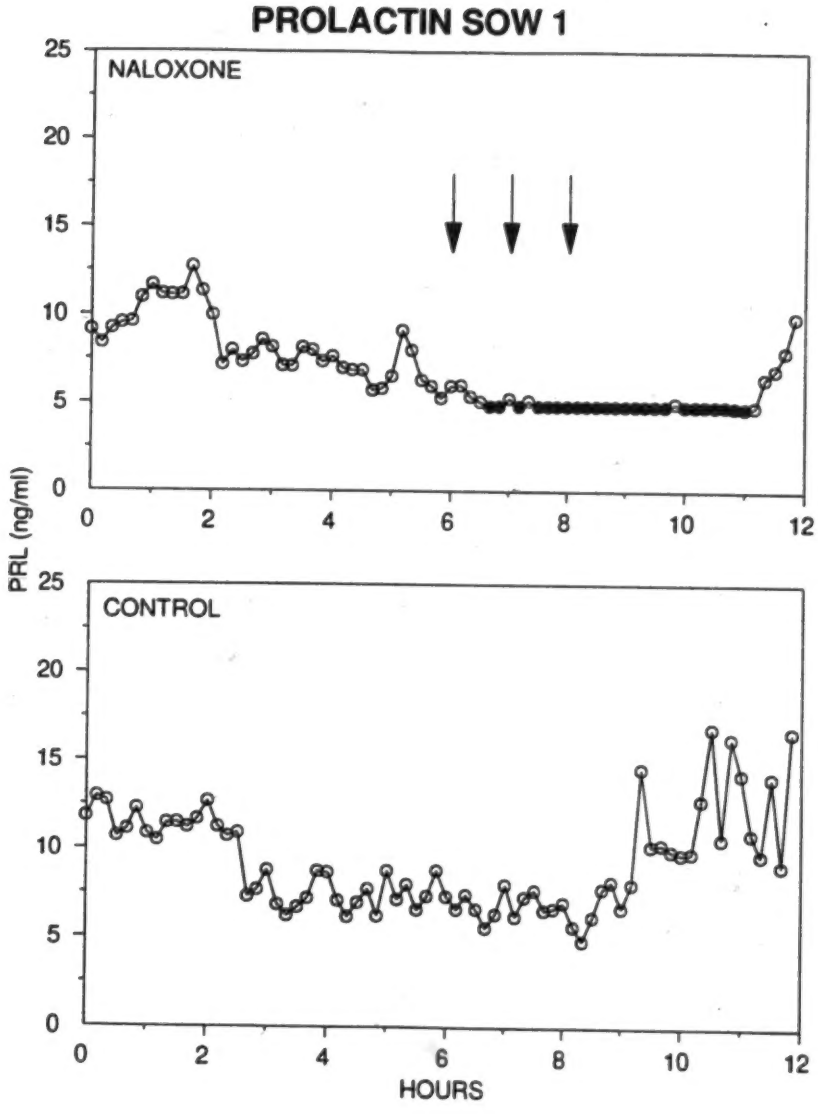

20
15
10
5
0
Mean PRL (ng/ml)
Control Day
Naloxone Day
a
c
a
a
c
b
1
2
3
4
Period of Sampling
Within Day
Control Period
Treated Period
(Control or Naloxone)

FIGURE 3.5 Plasma PRL profiles on control and naloxone days for two individual sows. Arrows indicate naloxone injections (2 mg/kg, 1 mg/kg, 1 mg/kg, respectively). Solid circles indicate values that were below the sensitivity of the assay. Note parabolic shape of profiles on control days.


PROLACTIN SOW 1
NALOXONE
CONTROL
PRL (ng/ml)
HOURS
0
5
10
15
20
25
0
2
4
6
8
10
12

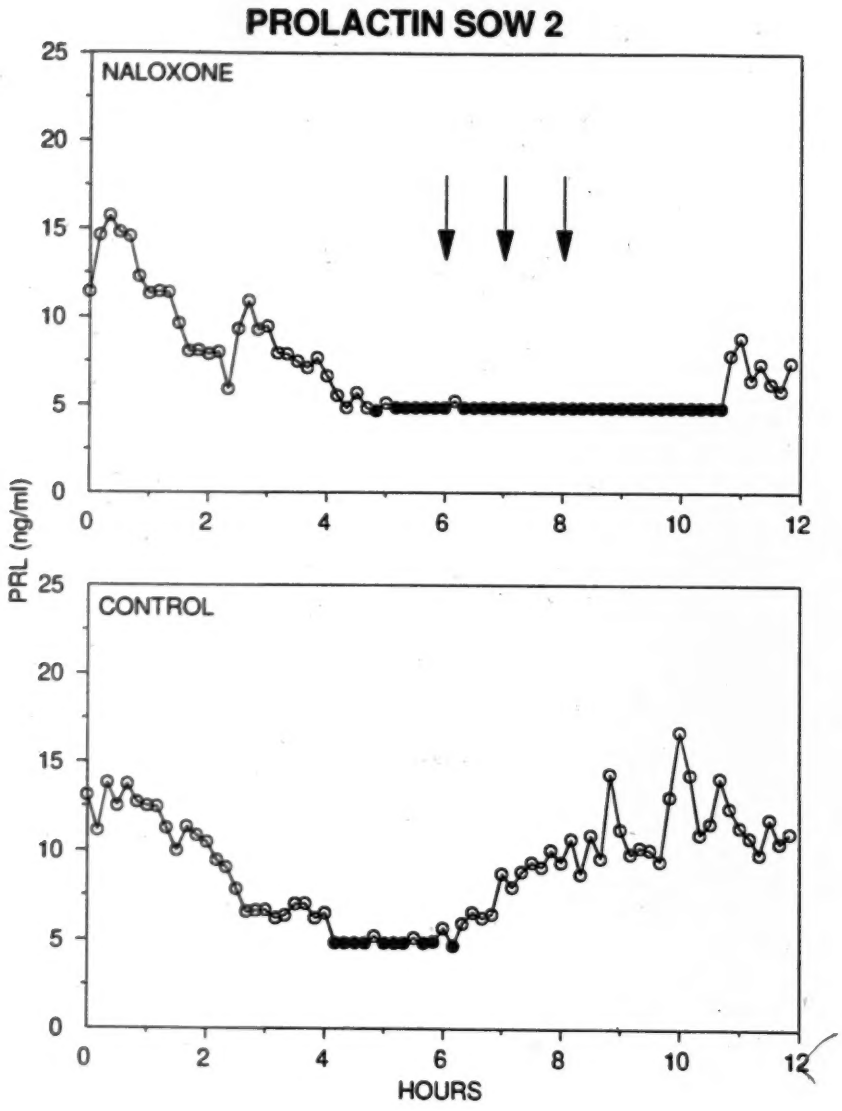

PROLACTIN SOW 2
NALOXONE
CONTROL
PRL (ng/ml)
HOURS
0
5
10
15
20
25
0
2
4
6
8
10
12

FIGURE 3.6 The relationship between mean plasma LH and PRL concentrations; a) the correlation between plasma LH and PRL concentrations throughout the entire 12-h control day; b) the correlation between plasma LH and PRL concentrations during the 6-h control period (Period 3) of the naloxone day; and c) following treatment with naloxone, the relationship between LH and PRL secretion is abolished.

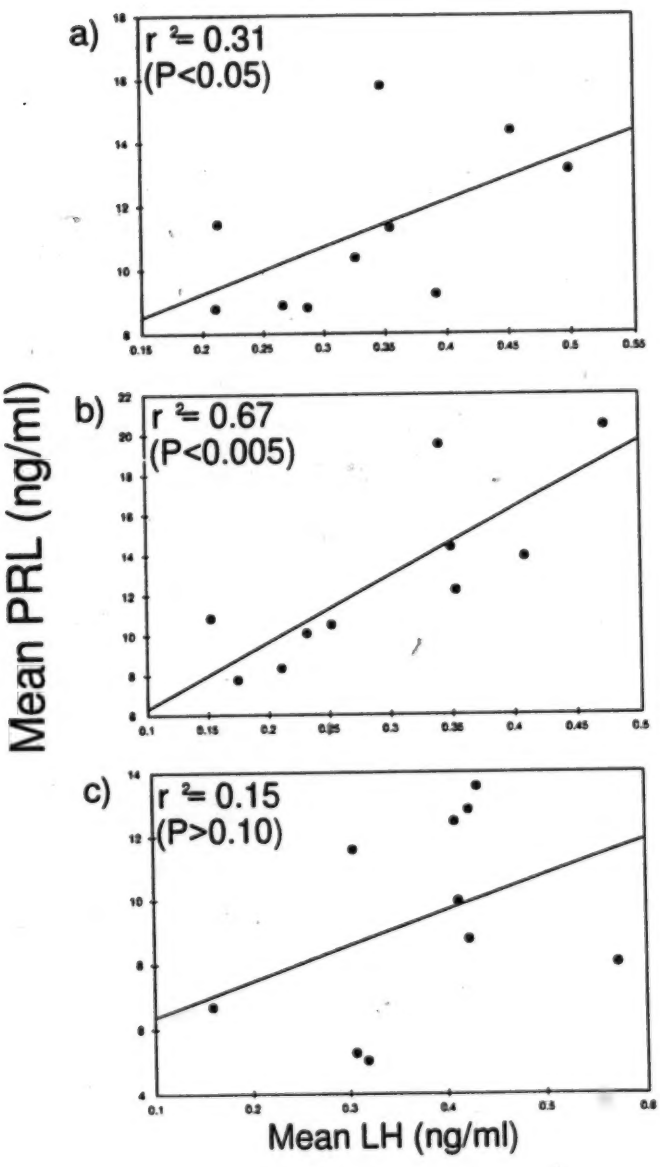

a)
r ² = 0.31
(P<0.05)
b)
r ² = 0.67
(P<0.005)
c)
r ² = 0.15
(P>0.10)
Mean PRL (ng/ml)
Mean LH (ng/ml)

FIGURE 3.7 Mean plasma PRL profiles for 10 sows on control (circles) and naloxone (squares) days. Arrows indicate naloxone injections (2 mg/kg, 1 mg/kg, 1 mg/kg, respectively). Both days had a significant polynomial regression ($P<0.0001$); however, naloxone delayed the expected afternoon increase in PRL secretion by almost 4 hours and the solid circle and square denote the computed absolute nadir of PRL secretion on control (1240h) and naloxone (1630h) days, respectively.

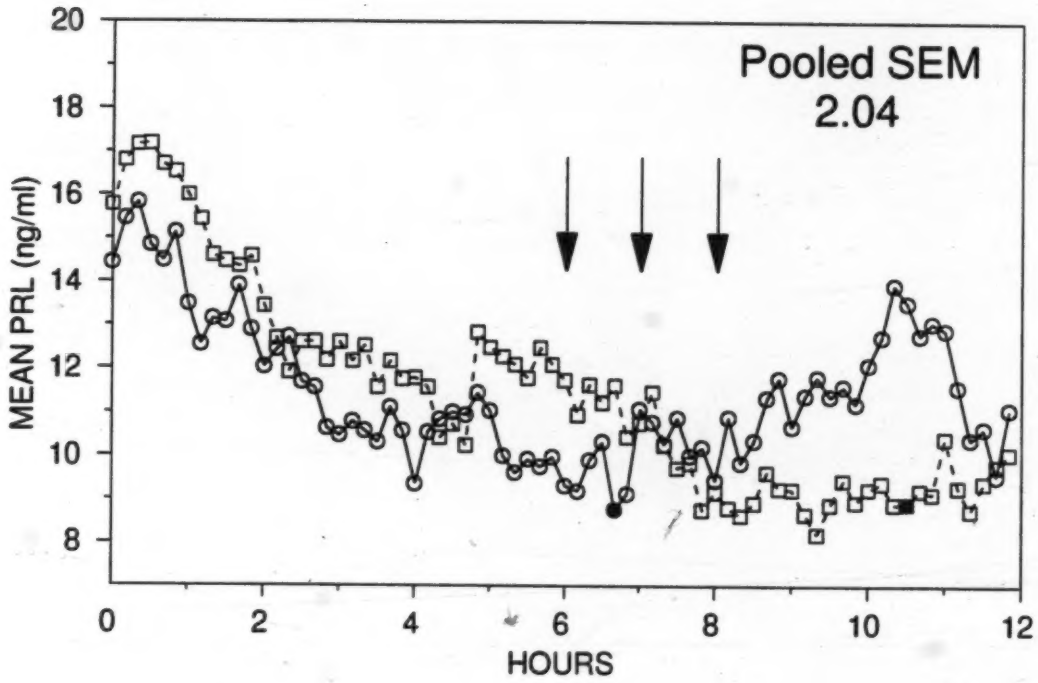
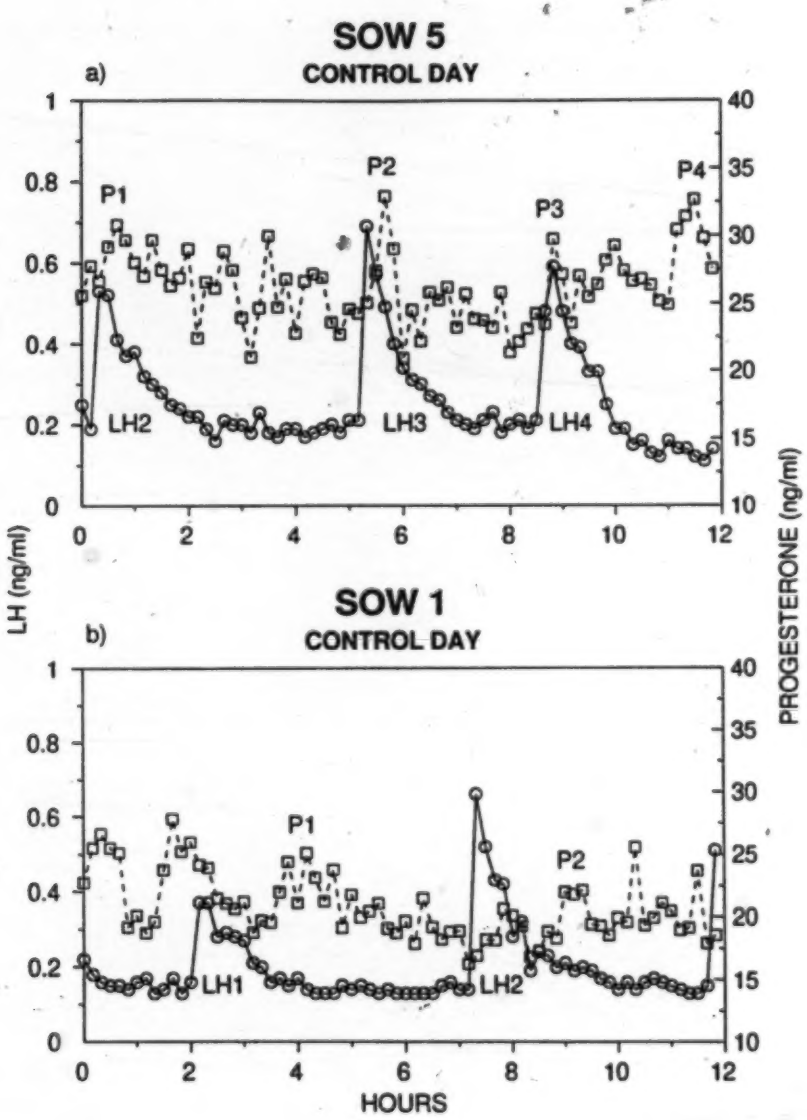

Pooled SEM
2.04
MEAN PRL (ng/ml)
20
18
16
14
12
10
8
0
2
4
6
8
10
12
HOURS

FIGURE 3.8 Time lag analysis of the relationship between LH (open circles) and progesterone (open squares). a) A sow in which a significant positive correlation was established with an estimated lag of zero minutes between the two hormones. It is suggested that the increase in progesterone (P1) is driven by an increase in LH (LH1) which occurred prior to the start of sampling. The lag between hormone secretion causes LH2 to occur simultaneously with P1 giving an apparent lag time of zero minutes. b) Sow 1; a sow in which a significant positive correlation was established with an estimated lag time of 100 minutes. c) Sow 2; a sow in which no positive correlation between the two hormones was established at any time lag.


SOW 5
a)
CONTROL DAY
P1
P2
P3
P4
LH2
LH3
LH4
SOW 1
b)
CONTROL DAY
P1
P2
LH1
LH2
LH (ng/ml)
PROGESTERONE (ng/ml)
HOURS

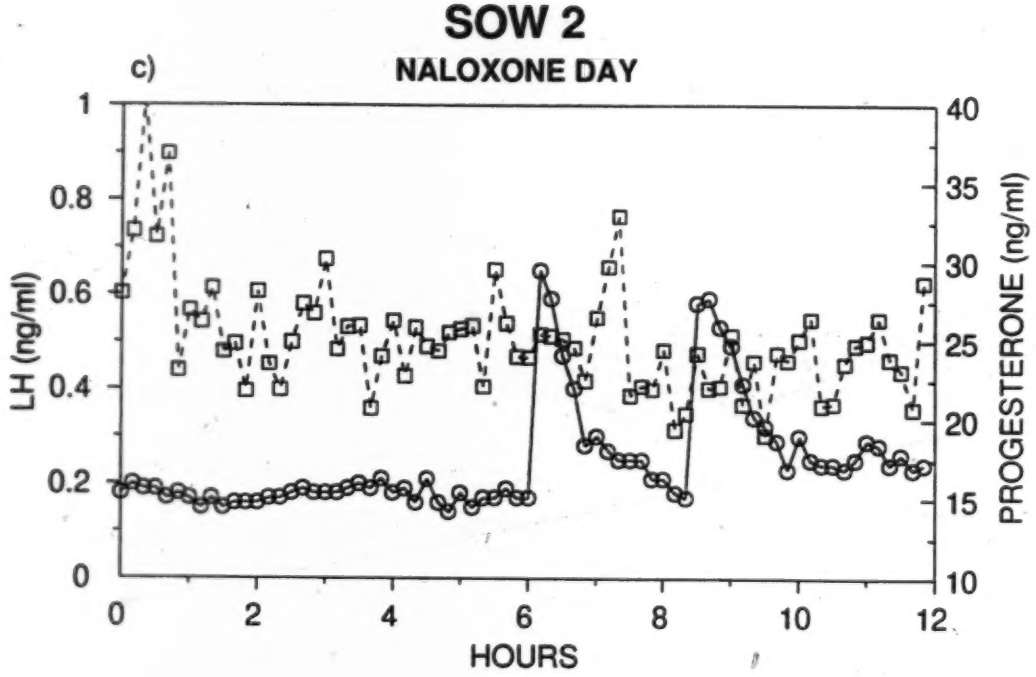

SOW 2
c)
NALOXONE DAY
LH (ng/ml)
PROGESTERONE (ng/ml)
HOURS
0
0.2
0.4
0.6
0.8
1
10
15
20
25
30
35
40
0
2
4
6
8
10
12

TABLE 3.1 LH mean, and mean estimates of maximum and minimum LH concentrations, and number of LH episodes (and the respective SEM) for each 6-h period, and the effect of naloxone treatment in Period 4. Values within a row with different subscripts differ ($P < 0.02$); ^{a,b} comparison within day; ^{c,d} comparison across day.

LUTEINIZING HORMONE (LH) CHARACTERISTIC:	TREATMENT DAY			
	CONTROL		NALOXONE	
	Period 1	Period 2	Period 3	Period 4
LH Mean	0.28 (0.02)	0.29 (0.02)^{c}	0.29 (0.03)^{a}	0.38 (0.03)^{b,d}
LH Maximum	0.36 (0.04)	0.38 (0.03)^{c}	0.38 (0.04)^{a}	0.50 (0.05)^{b,d}
LH Minimum	0.21 (0.02)	0.21 (0.02)^{c}	0.22 (0.02)^{a}	0.27 (0.02)^{b,d}
Number of Episodes	1.78 (0.20)	1.75 (0.23)	2.00 (0.35)	2.28 (0.14)

TABLE 3.2 Significant correlations, and their associated time lags, on control and naloxone treated days between LH and progesterone secretion in 10 sows during late gestation.

Sow	Treatment	Correlation	P Value	Lag (min)
1	control	0.29	0.02	100
1	naloxone	0.31	0.007	0
		-0.32	0.008	50
2	control	-0.38	0.001	40
2	naloxone	-0.39	0.002	80
3	control	0.25	0.04	40
3	naloxone	0.46	0.0001	20
		-0.32	0.02	150
4	control	0.26	0.03	0
		-0.35	0.004	50
4	naloxone	0.25	0.03	0
5	control	0.25	0.03	10
5	naloxone	0.31	0.01	40
6	control	0.27	0.03	80
6	naloxone	0.28	0.03	100
7	control	0.47	0.0001	110
7	naloxone	-0.27	0.02	20
8	naloxone	-0.40	0.0006	10
9	control	0.41	0.001	130
		-0.32	0.008	50
9	naloxone	-0.37	0.004	120
10	control	0.28	0.03	120
10	naloxone	0.24	0.04	10
		-0.32	0.01	140

CHAPTER 4
Noradrenergic/Opioidergic Interactions Controlling LH Secretion in Lactation and at Weaning in the Sow[1]

INTRODUCTION

There is ample evidence that in rats (Nowak and Swerdloff, 1985; Leung *et al.*, 1982; Kinoshita *et al.*, 1981; Jarry *et al.*, 1990; Martin *et al.*, 1995) and primates (Kaufman *et al.*, 1985; Terasawa *et al.*, 1988; Gearing and Terasawa, 1991) norepinephrine stimulates GnRH secretion from the hypothalamus and that these effects are mediated by α_1 adrenoreceptors. It is also well documented in the rat that endogenous opioid peptides act through inhibition of presynaptic stimulatory adrenergic pathways to inhibit GnRH, and hence LH, secretion (Miller *et al.*, 1985; Diez-Guerra *et al.*, 1987; Dyer and Grossmann, 1988; Clough *et al.*, 1990; Dyer *et al.*, 1991; Nishihara *et al.*, 1991).

In contrast, in the pig there is a relative paucity of information on the neuroendocrine regulation of the GnRH pulse generator, although this information is fundamental to an evaluation of potential clinical approaches to enhancing fertility. A number of laboratories have shown that opioid peptides are involved in the inhibition of LH secretion during established lactation in the sow (Barb *et al.*, 1986; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a and 1988b; De Rensis, 1993; De Rensis *et al.*, 1993a). After weaning this opioidergic inhibition is removed (Barb *et al.*, 1986; Armstrong *et al.*, 1988a and 1988b) and LH secretion increases dramatically in many sows (for reviews see Edwards, 1982; Varley and Foxcroft, 1990; Foxcroft *et al.*, 1995). During lactation endogenous opioids appear to act at the hypothalamic level to decrease GnRH release, as exogenous GnRH administration can overcome opioidergic inhibition of LH secretion (Barb *et al.*, 1986; Sesti and Britt, 1993; De Rensis, 1993). Furthermore, Barb *et al.* (1994) have recently provided evidence for opioidergic modulation of GnRH release from hypothalamic preoptic area explants in the pig. Barb *et al.* (1990) have also demonstrated *in vitro* that β-endorphin may interfere with gonadotrophin secretion at the pituitary level. Kineman *et al.* (1988 and 1989) identified GnRH perikarya primarily in the preoptic area and GnRH axons extending to the median eminence. They also identified proopiomelanocortin immunoreactive neurons within the same regions of the brain. A similar study identified tyrosine hydroxylase immunopositive neurons in the same area as GnRH neurons (Leshin *et al.*, 1989). These data provide an anatomical basis for interactions between opioidergic and noradrenergic systems in the regulation of GnRH secretion in the pig. Kesner *et al.* (1987) and Chang *et al.* (1989 and 1993) have shown that noradrenergic synthesis inhibitors can suppress LH secretion in the cyclic and ovariectomized gilt and it appears that opioid peptides may regulate norepinephrine and GnRH secretion (Chang *et al.*, 1993). However, in the lactating sow there is no evidence that endogenous opioids released within the hypothalamus act at the pre-synaptic level to block

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[1] A shorter version of this paper has been submitted to 'Biology of Reproduction'.

stimulatory catecholamines or directly on the GnRH neuron (reviewed by Foxcroft, 1992). Therefore, the objective of this study was to determine if opioid peptides exert their influence pre-synaptically, at the level of the noradrenergic neuron, or post-synaptically, at the level of the GnRH neuron, in the lactating and weaned sow.

MATERIALS AND METHODS

Animals and Blood Collection

A total of 16 primiparous Camborough PIC crossbred sows from the University of Alberta Swine Research Unit were used in two replicates. Sows were housed in farrowing crates from d109 of gestation until weaning. Water and feed were provided on an *ad lib* basis and a lighting regimen of 12L:12D, lights on at 0600h, was maintained throughout lactation. Average (± S.D.) weight of the sows was 192.8 ± 18.9 kg and sows suckled between 7 and 11 (mean (± S.D.) 8.63 ± 1.20) piglets during the experimental period. Sows underwent surgery between days 3-6 post-partum (farrowing = d0) for placement of indwelling jugular vein catheters via the cephalic vein.

In part one of the experiment 3 ml blood samples were taken at 10 min intervals between 0600-1800h on alternate days between d7 and d11 of lactation, in a repeat measures design, so that all sows received all treatments once. The first 6 h of each day was considered a within day control period, while the subsequent 6 h was the treatment and trail-off period (Fig.4.1). Sows were weaned between d19 and d27 of lactation as a group at 2400h and in part 2 of the experiment, samples were again collected at 10 min intervals for 12 h from 0600h the next day (Fig.4.2). Blood samples were collected into heparinized tubes, centrifuged at 1500 x g at 4°C for 15 min and the plasma frozen at -30°C until assayed for LH. Catheters were flushed with 2ml heparinized saline (10 I.U./ml) after each blood sample.

Treatments

The α_1-noradrenergic receptor agonist, phenylephrine, was first used in a dose response study, using separate lactating animals from that in the main study, to establish that a dose considered adequate to affect LH release would not result in any adverse behavioural or physiological side effects.

In part 1 of the study, during established lactation, three treatments were randomized over alternate sampling days. Treatment N consisted of 2 mg/kg naloxone hydrochloride (Sigma Chemical Co., St. Louis, MO; 50 mg/ml in sterile saline) administered intravenously at 1200h, followed by 2 further injections of 1 mg/kg at hourly intervals. Treatment P consisted of 25 µg/kg of phenylephrine hydrochloride (Sigma; 1000 µg/ml in sterile saline) administered intravenously at 1200h followed by two further 25 µg/kg injections at hourly intervals. In treatment NP, N was given first, immediately followed by P, at the intervals, doses and route of administration stated previously (Fig.4.1). As there is little evidence to suggest a diurnal rhythm in LH secretion during established lactation (Barb *et al.* , 1986; Mattioli *et al.* , 1986; Armstrong *et al.* , 1988a; De Rensis, 1993; De Rensis *et al.* , 1993a and 1993b), neither a control day or afternoon control period was included in the treatment groups.

In part 2 of the study, treatments were randomly allocated to sows before weaning. After weaning, treatment group M (n=6) received a 0.1 mg/kg intravenous injection of morphine sulphate (15 mg/ml; Sabex, Montreal, Canada) at 1200h followed by two further 0.1 mg/kg injections at hourly intervals, a treatment regimen known to replace opioid tone and inhibit LH without adverse behavioural side effects (De Rensis, 1993). Group MP (n=6) received both 0.1 mg/kg morphine and 25 μg/kg phenylephrine intravenously at 1200, 1300 and 1400h, to evaluate the ability of phenylephrine to overcome the opioidergic inhibition of LH secretion. Group C (n=4) were controls and received no treatment. The first 6-h period of sampling was used to establish a pretreatment LH baseline in all sows after weaning and to ensure no differences in pretreatment LH secretion between groups; the second 6-h period of sampling in C sows was used to confirm that any changes in LH secretion were due to treatment (Fig.4.2).

Hormone Assays

Plasma LH was quantified in all samples using the double-antibody RIA described by De Rensis *et al.* (1993b). The purified porcine LH used for iodination and standards were kindly supplied by Dr. J.H.F. Erkens (Research Institute for Animal Production, Ziest, Netherlands) and Dr. S.D. Glenn (Alton Jones Cell Science Center, Lake Placid, NY, USA), respectively. The intra- and inter-assay coefficients of variation were 5.6% and 8.2%, respectively. The overall sensitivity of the assays, defined as 80% of total binding, was 0.01 ng/tube.

Statistical Analysis

As in previous experiments (De Rensis, 1993; Willis *et al.*, 1996), LH period means rather than LH pulse characteristics were used to analyse effects of treatments because naloxone produced confounding effects on episodic LH activity in lactation. The pattern of plasma LH concentrations after weaning also negated use of pulsatile analysis. However, LH pulse frequency, where appropriate, was visually appraised using the criteria established by Cosgrove *et al.* (1991). Maximum and minimum values for LH data were also analysed using the statistical models described below. Analysis of the data indicated that treatment effects on mean, maximum and minimum LH characteristics were similar, therefore only data for mean LH is presented.

Part 1: For the purpose of statistical analysis, data for each 12-h sampling block was split into two 6-h periods for a total of 6 periods over 3 days of sampling; periods 1, 3 and 5 acted as within day controls and irrespective of original order of treatment, periods 2, 4 and 6 were designated as N, P and NP treated periods, respectively. All data were analysed using the PROC GLM procedure of SAS (SAS, 1988).

Preliminary analysis of control period data confirmed that order of treatment did not affect (P=0.51) mean LH, however day of lactation appeared to influence LH secretion (P=0.09). Therefore, although originally designed as a repeated measures analysis, due to partial missing data for one sow and the almost significant effect of day of lactation, data were treated as an incomplete randomized block, and covariate analysis was used to determine effects of treatment on LH secretion in periods 2, 4 and 6. Replicate, sow and treatment were

used as the main class variables and mean LH for the within day control period (periods 1, 3 and 5) was used as the covariate. Where appropriate, differences among treatments were compared by Student-Neuman-Keuls (SNK) test.

Part 2: The 12-h post-weaning sampling block was split into two periods, a 6-h pre-treatment control period and a 6-h treatment and trail off period. Period LH means were analysed using treatment as the main class variable. In the event of a significant overall treatment effect, differences between treatment means were determined by least squares contrasts.

RESULTS

Part 1:

On the N, P and NP treated days during lactation, LH means for the within day control periods were, 0.208, 0.249 and 0.242 ng/ml, respectively. There was an overall treatment effect ($P<0.008$) on LH secretion (Fig.4.3). Treatment with N and NP increased mean LH compared to treatment with P alone ($P<0.02$; mean (± SEM) LH 0.28 ± 0.03, 0.30 ± 0.04 and 0.21 ± 0.03 ng/ml, respectively) but the response to N and NP were not different ($P>0.05$). The LH response to naloxone treatment is clearly apparent from the individual LH profiles presented in Figure 4.4. Compared to low LH pulsatility in the control periods, LH pulsatility increased in response to naloxone and naloxone/phenylephrine combined. If anything, phenylephrine appeared to inhibit episodic LH secretion, although the final model of co-variate analysis for LH precluded direct analysis of this effect on mean LH concentrations.

Part 2:

After weaning, there was no difference ($P=0.85$) between control period LH concentrations for the three treatment groups and mean (± SEM) LH concentrations across all sows was 0.48 ± 0.04 ng/ml. There was also no difference in mean LH in the first and second 6-h period of sampling for the control sows ($P=0.36$), indicating that estradiol negative feedback had not yet occurred. Mean LH for M, MP and C treatment periods were 0.22, 0.25 and 0.48 ng/ml, respectively (Fig.4.5). M and MP significantly suppressed LH compared to C ($P<0.02$ and $P<0.04$, respectively) but M and MP were not different ($P=0.72$). Reference to Figure 4.6 clearly confirms that morphine treatment exerted an immediate and dramatic inhibitory effect on the very active pattern of LH secretion in the immediate period after weaning and that this inhibitory opioidergic effect was not negated by concomitant treatment with phenylephrine.

DISCUSSION

In this study we have confirmed that endogenous opioids suppress LH secretion during established lactation in the sow and that this inhibition is removed at weaning. These findings are consistent with several other studies in the lactating (De Rensis 1993; De Rensis *et al.*, 1993a, Barb *et al.*, 1986; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a) and weaned (Barb *et al.*, 1986, Armstrong *et al.*, 1988a and 1988b) sow. Although our conclusions are

based on changes in mean LH concentrations, where appropriate, analysis of LH pulsatility confirmed that the LH responses observed were clearly associated with treatment effects on the pattern of LH pulsatility (Tables 4.1 and 4.2).

Several studies in the rat and primate, using electrical and neurotoxic lesioning (Clifton and Steiner, 1985; Jarry *et al.*, 1990; Leonhardt *et al.*, 1991), electrical recordings (Kaufman *et al.*, 1985; Condon *et al.*, 1989), synthesis inhibitors (Kinoshita *et al.*, 1981; Martin *et al.*, 1995) and various agonists and antagonists (Leung *et al.*, 1982; Nowak and Swerdloff, 1985; Terasawa *et al.*, 1988; Gearing and Terasawa, 1991), have demonstrated that the noradrenergic system is involved in the generation of pulsatile GnRH/LH secretion, and that noradrenergic effects on GnRH neurons are mediated postsynaptically via α_1 noradrenergic receptors. Further studies in the rat have shown that opioidergic inhibition of GnRH/LH involves presynaptic inhibition of the stimulatory noradrenergic drive (Diez-Guerra *et al.*, 1987; Dyer and Grossman, 1988; Clough *et al.*, 1990; Dyer *et al.*, 1991). Morphine, ß-endorphin and met-enkephalin, but not dynorphin A (1-8), inhibited electrically stimulated release of ^{3}H-noradrenaline from perifused slices of rat preoptic area, and the effects of these opioid agonists were reversed by the addition of naloxone to the medium (Diez-Guerra *et al.*, 1987). Electrical recordings of medial preoptic area neurons showed that stimulation of the ventral noradrenergic tract stimulated these neurons. Iontophoretic application of naloxone further enhanced neuronal firing, while morphine reversed the effects of naloxone or enhanced inhibition (Dyer and Grossmann, 1988). Opioidergic binding and norepinephrine content in the preoptic area and anterior hypothalamus decreased following electrolytic and neurochemical lesioning of the ventral noradrenergic tract in ovariectomized steroid treated rats (Dyer *et al.*, 1991). Clough *et al.* (1990) has shown that opioidergic receptor blockade with naloxone acts synergistically with phenylephrine to increase release of GnRH from preoptic area-mediobasal hypothalamic explants from intact adult female rats. Similarly, opioidergic blockade using naloxone increased hypothalamic multiunit activity, which was associated with LH pulses, but administration of the α_1 antagonist, phenoxybenzamine, abolished both electrical activity and LH pulsatility (Nishihara *et al.*, 1991). Although most literature indicates a presynaptic inhibition of GnRH by opioids, recently Lagrange *et al.* (1995) have demonstrated post-synaptic effects of μ-receptor agonists on tetrodotoxin isolated GnRH neurons in ovariectomized guinea pigs.

There is little information regarding noradrenergic, opioidergic and GnRH interactions in the sow. A role for opioidergic inhibition of GnRH/LH secretion in the pig has been firmly established, as previously discussed. However, opioidergic effects on systems impinging on GnRH or directly on GnRH neurons are little understood. Parvizi and Ellendorff (1978 and 1982) demonstrated that the noradrenergic system may be involved in LH secretion in studies in which norepinephrine administered intraventricularly to male Göttingen miniature pigs resulted in significantly elevated plasma LH. However, norepinephrine microinjected into several different brain nuclei of ovariectomized miniature pigs resulted in stimulatory or inhibitory effects on LH secretion dependent on the brain region and dose. Leshin *et al.* (1989) identified tyrosine hydroxylase immunopositive neurons in the hypothalamic-preoptic area of swine; however, there was no mention of the animals sex or reproductive condition and there was no attempt to distinguish between noradrenergic or dopaminergic neurons that

may have been stained positively. Chang *et al.* (1990) administered diethyldithiocarbamate (DDC), a noradrenergic synthesis inhibitor to ovariectomized gilts. DDC suppressed mean and basal LH secretion and delayed the estradiol induced LH surge suggesting that a noradrenergic drive was important to maintaining LH secretion in these animals. In a preliminary study using rats, Chang *et al.* (1992) compared N-methyl-N^{-1}[1-methyl-2-propenyl] 1,2 hydrazine-dicarbothioamide (AIMAX), a carbamate with gonadotropin inhibiting properties (Kesner *et al.*, 1987), with DDC. They found that like DDC, AIMAX also reduced norepinephrine content of the mediobasal hypothalamus. This group (Chang *et al.*, 1993) has since used AIMAX in steroid treated ovariectomized gilts to suppress LH secretion and show that opioidergic antagonism with naloxone can not increase LH secretion in AIMAX treated animals. From these findings they have suggested that opioids act within the hypothalamus to inhibit noradrenergic stimulatory activity.

These results indicate that phenylephrine, a specific α_1-noradrenergic receptor agonist, given intravenously, was unable to stimulate GnRH/LH secretion during established lactation, and hence overcome opioidergic inhibition, in the sow. Lack of a phenylephrine response may have been due to suppression of LH secretion by a non-opioidergic mechanism. However, the timing of our experiment is considered to be appropriate, as De Rensis (1993) and De Rensis *et al.* (1993a) have shown that an opioidergic mechanism is in place to suppress GnRH/LH secretion by 72 hours post-partum. Indeed, the LH secretory response to opioidergic antagonism with naloxone was comparable to that seen in previous studies with the lactating sow (Barb *et al.*, 1986; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a; De Rensis, 1993; De Rensis *et al.*, 1993a). The dose of phenylephrine used may have been insufficient to overcome opioidergic inhibition of GnRH/LH, however our initial study indicated that higher intravenous doses could not be used without causing adverse behavioural side effects. Nevertheless, even this dosage, delivered peripherally, may have allowed phenylephrine access to GnRH neuronal terminals which lie outside the blood brain barrier but not to GnRH perikarya which may be critical for phenylephrine to stimulate a secretory response.

Plasma LH concentrations after weaning in this study were comparable with those reported by Foxcroft *et al.* (1987). In the rat, morphine acts to prevent increases in norepinephrine turnover and inhibits the steroid-induced LH surge in ovariectomized rats, and naloxone can prevent these actions (Akabori and Barraclough, 1986). Consistent with the results of Armstrong *et al.* (1988b) who used morphine to suppress the acute increase in LH secretion in response to transient weaning of sows from their litters, morphine suppressed LH secretion. However, phenylephrine coadministered with morphine after weaning was unable to overcome this morphine-induced inhibition of GnRH/LH secretion, indicating that at the dose used, intravenous administration of phenylephrine could not overcome the opioidergic inhibition of the GnRH neuron. Combined phenylephrine and ß-endorphin treatment of rat preoptic area-mediobasal hypothalamic explants resulted in a biphasic pattern of GnRH secretion (Clough *et al.*, 1990). These authors suggest that the biphasic response was due to differential or independent effects of opioids and norepinephrine on GnRH release, because if the opioids functioned only to inhibit norepinephrine, then ß-endorphin should have had no transient inhibitory effect on the stimulatory action of phenylephrine. Miller *et al.* (1985) reached similar conclusions, as naloxone could still stimulate LH release in male rats in which

the ascending noradrenergic tract had been cut, and phenoxybenzamine, an alpha blocker, was ineffective in preventing the naloxone induced rise in LH. It appears that in certain cases opioid peptides do not require a functional noradrenergic system to inhibit GnRH secretion, and opioidergic receptors directly on GnRH neurons can not be ruled out.

It is possible in the sow that phenylephrine was unable to overcome opioidergic inhibitory actions because a noradrenergic drive is only facilitory to GnRH/LH secretion. Another more important stimulatory system may be involved which is presynaptically inhibited by opioids. The results of Miller *et al.* (1985) do not rule out the possibility that there is a stimulatory interneuron being inhibited by endogenous opioids. Several studies in the rat indicate that even after chronic removal of norepinephrine from the hypothalamus there is another system functioning to drive the GnRH pulse generator, as evinced by resumed pulsatile LH secretion and the inability of noradrenergic antagonists to disrupt LH secretion (Herdon *et al.*, 1984; Clifton and Steiner 1985; Leonhardt *et al.*, 1991). However, the identity of this alternate system remains unknown.

We therefore suggest four possible explanations for the inability of the exogenously administered noradrenergic agonist, phenylephrine, to overcome either endogenous or exogenous opioidergic inhibition of GnRH/LH secretion: 1) that the intravenously administered dosage of phenylephrine may have allowed phenylephrine access to GnRH neuronal terminals but not to GnRH perikarya which may be critical for it to stimulate a secretory response, 2) that in the sow an α_1-noradrenergic receptor is not involved in stimulating GnRH secretion, 3) that an alternate, more important, stimulatory input to GnRH secretion is inhibited by the endogenous opioids, or 4) that opioid peptides act post-synaptically, directly on the GnRH neuron.

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FIGURE 4.1 Schematic diagram of the experimental design for Part 1, showing a representative treatment for a sow. 3-ml blood samples were taken at 10 min intervals between 0600-1800 on alternate days between d7 and d11 of lactation, in a repeat measures design, so that all sows received all treatments once. The first 6 h of each day was considered a within day control period, while the subsequent 6 h was the treatment and trail-off period. Treatment N consisted of 2 mg/kg naloxone administered intravenously at 1200h, followed by 2 further injections of 1 mg/kg at hourly intervals. Treatment P consisted of 25 µg/kg of phenylephrine administered intravenously at 1200h followed by two further 25 µg/kg injections at hourly intervals. In treatment NP, N was given first, immediately followed by P, at the intervals, doses and route of administration stated previously.

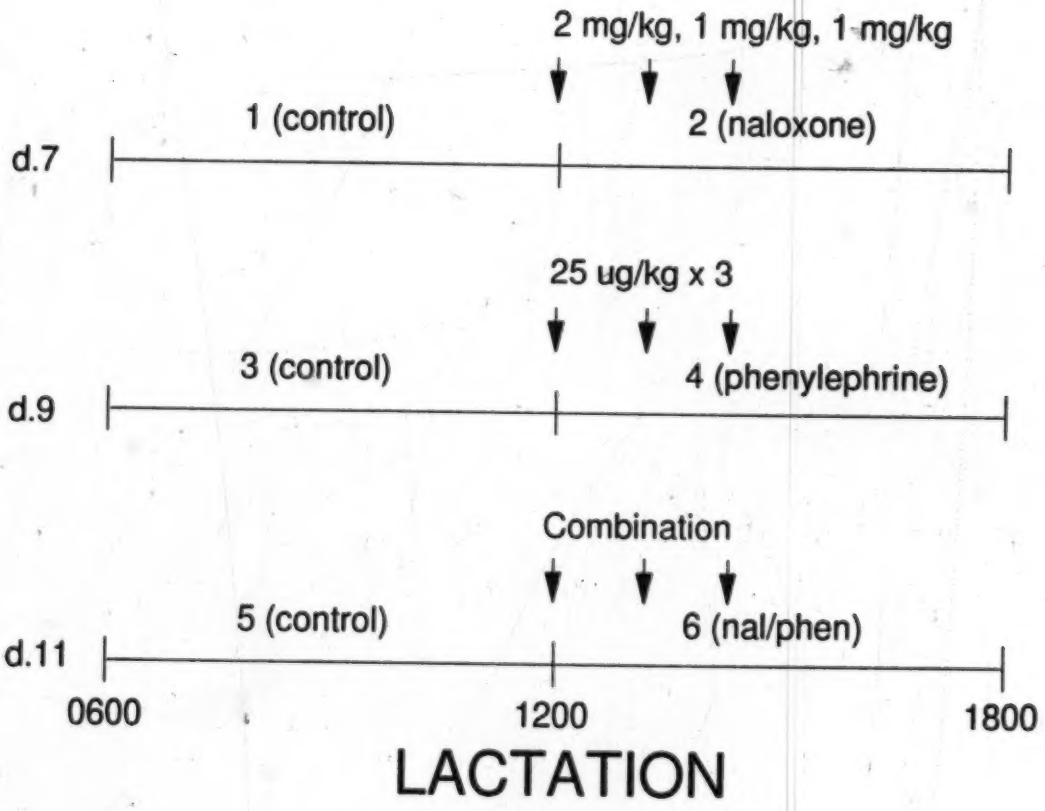
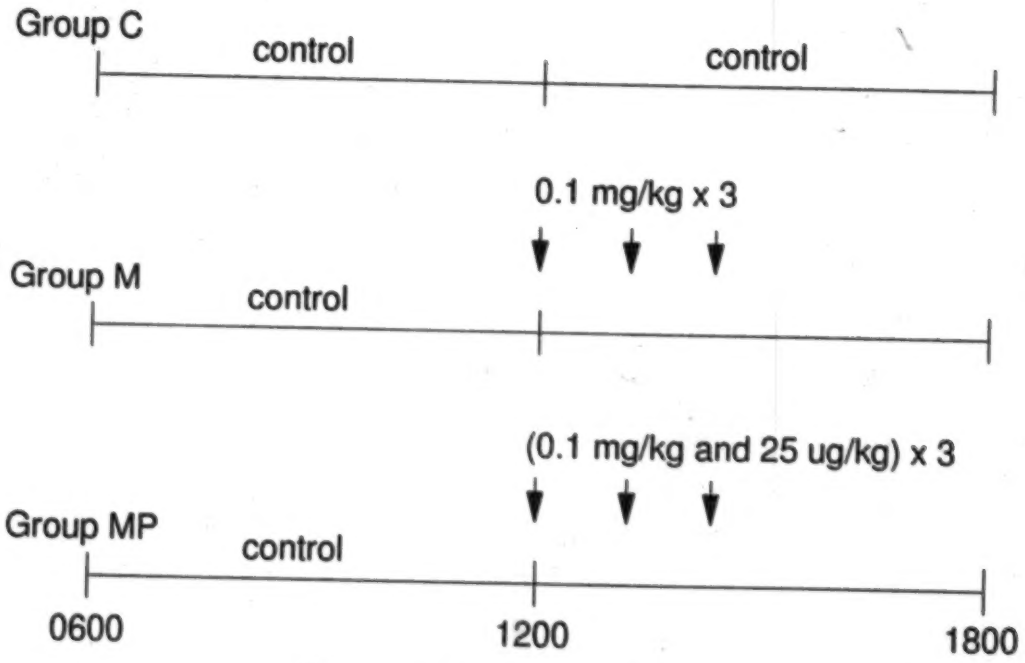

2 mg/kg, 1 mg/kg, 1 mg/kg
1 (control)
2 (naloxone)
d.7
25 ug/kg x 3
3 (control)
4 (phenylephrine)
d.9
Combination
5 (control)
6 (nal/phen)
d.11
0600
1200
1800
LACTATION

FIGURE 4.2 Schematic diagram of the experimental design for Part 2, post-weaning, showing the three treatment groups. The first 6-h period was used to establish LH concentrations for each sow after weaning and the second 6-h period was used to monitor the response to treatment. Treatment group C (n=4) were controls and received no treatment; group M (n=6) received a 0.1 mg/kg intravenous injection of morphine at 1200h followed by two further 0.1 mg/kg injections at hourly intervals; and group MP (n=6) received both 0.1 mg/kg morphine and 25 μg/kg phenylephrine intravenously at 1200, 1300 and 1400h.



Post-Weaning

FIGURE 4.3. Mean (+ SEM) plasma LH concentrations during lactation in a group of 16 sows. Periods 1, 3 and 5 represent within day control periods, and periods 2, 4 and 6 are naloxone (N), phenylephrine (P) and naloxone/phenylephrine (NP) treated periods, respectively. Data are presented irrespective of the order of treatment or day of lactation (see text). Superscripts ^{a,b} differ ($P<0.02$) for comparison of treatments across days.

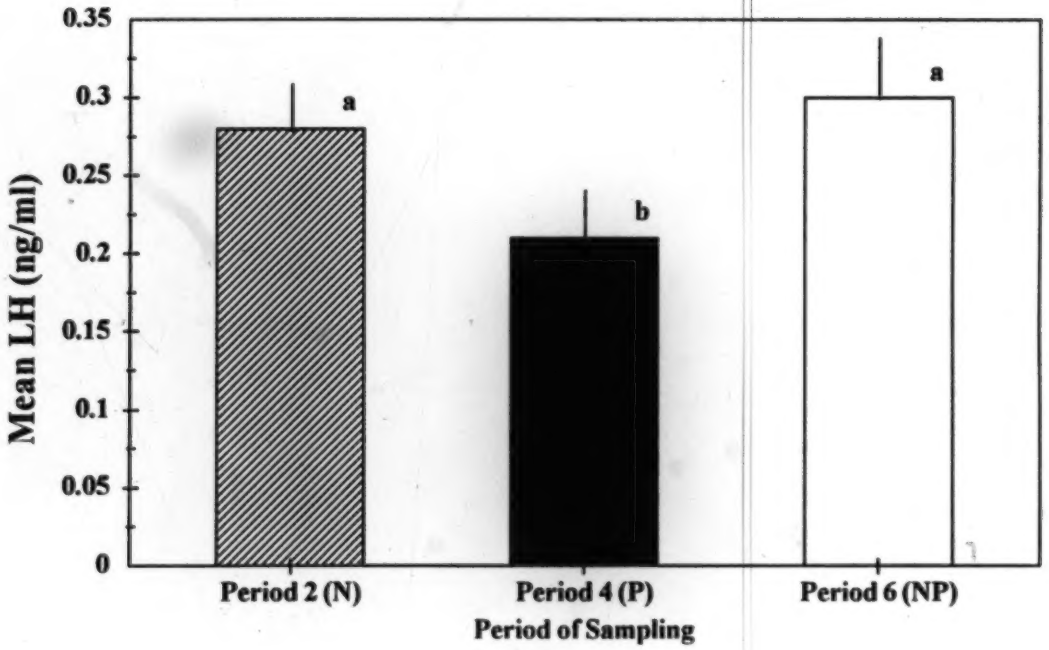
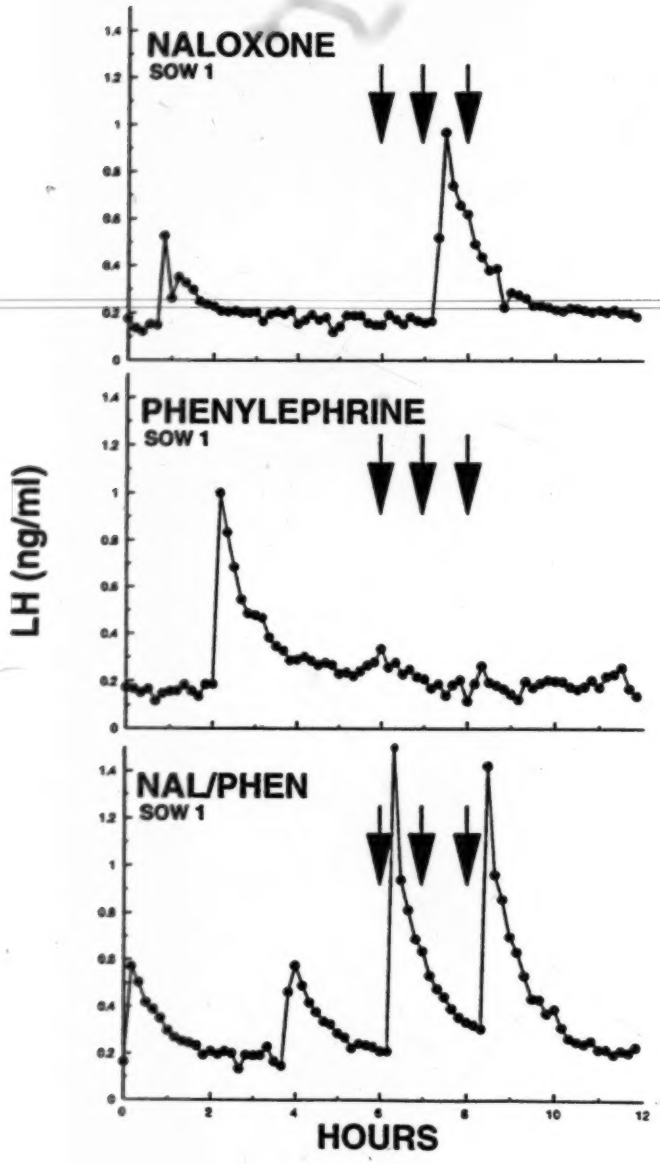

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Mean LH (ng/ml)
a
b
a
Period 2 (N)
Period 4 (P)
Period 6 (NP)
Period of Sampling

FIGURE 4.4 Individual plasma LH profiles of two sows. Arrows indicate time of treatment: naloxone (N; 2 mg/kg, 1 mg/kg, 1 mg/kg), phenylephrine (P; 25 μg/kg X 3) and combined naloxone/phenylephrine (NP; at doses stated previously).


NALOXONE
SOW 1
PHENYLEPHRINE
SOW 1
NAL/PHEN
SOW 1
LH (ng/ml)
HOURS

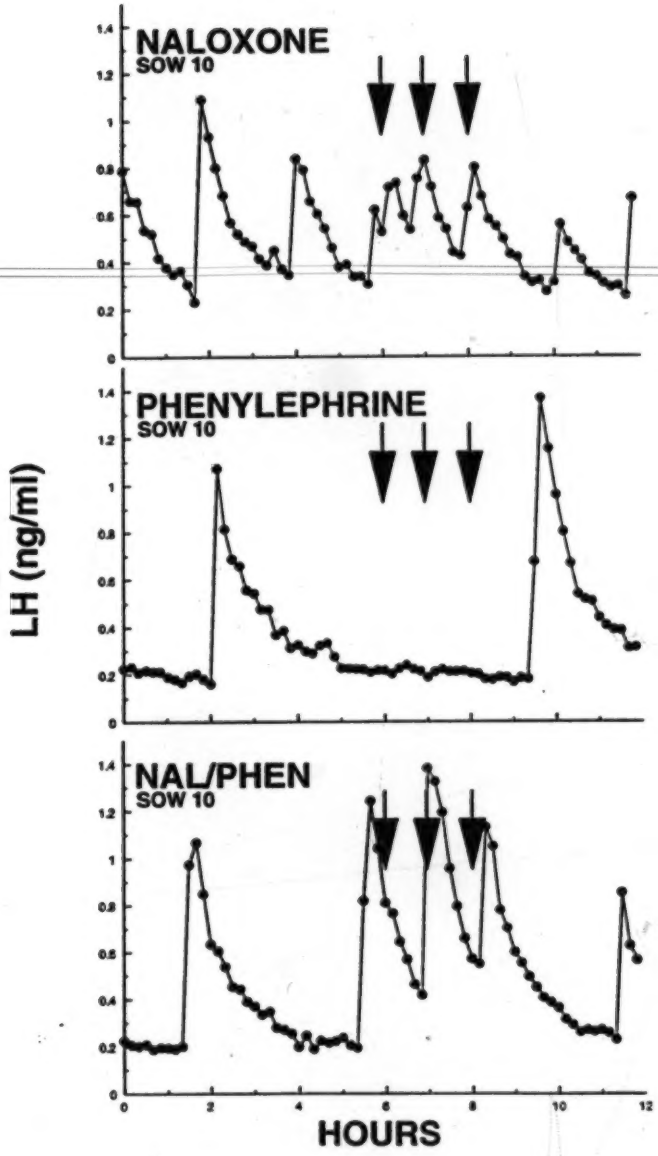

NALOXONE
SOW 10
PHENYLEPHRINE
SOW 10
NAL/PHEN
SOW 10
LH (ng/ml)
HOURS

FIGURE 4.5 Mean (+ SEM) plasma LH concentrations of 16 sows in the post-weaning period, 23.56 ± 2.48 days after farrowing. There was no significant differences among control periods (period 1) in LH secretion for each treatment group, or between the first and second 6-h period of sampling in Control sows. Superscripts ^{a,b} differ ($P<0.04$) for comparison of treatment periods (period 2).

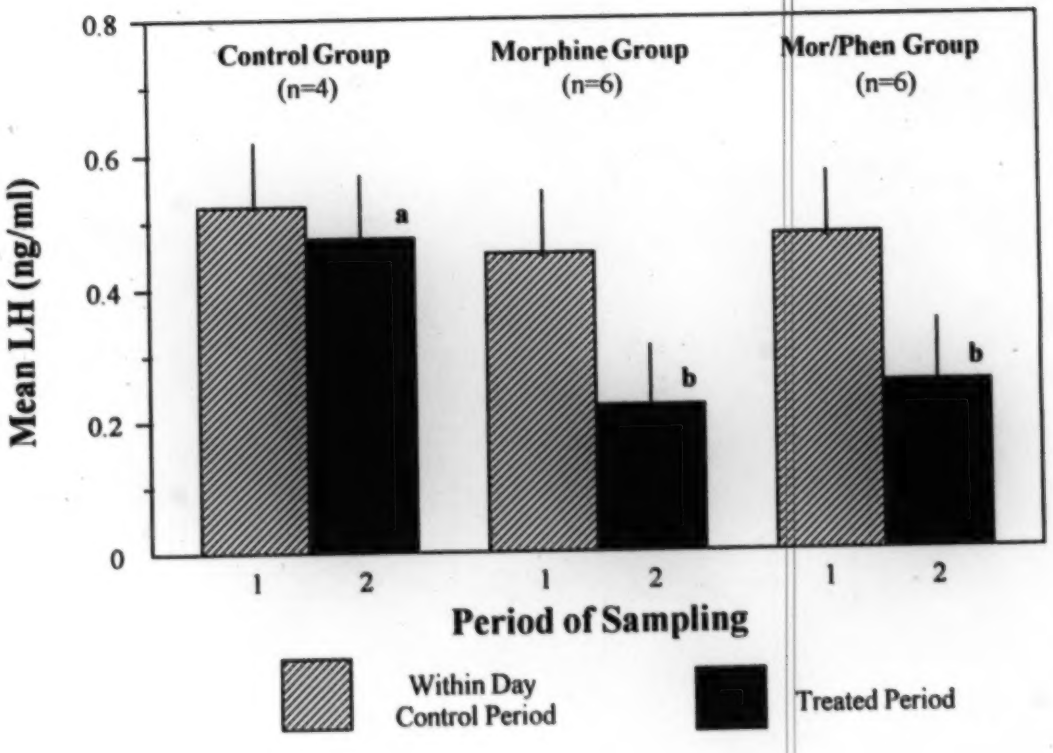

Control Group
(n=4)
Morphine Group
(n=6)
Mor/Phen Group
(n=6)
Mean LH (ng/ml)
0.8
0.6
0.4
0.2
0
a
b
b
1
2
1
2
1
2
Period of Sampling
Within Day
Control Period
Treated Period

FIGURE 4.6 Individual plasma LH profiles representing each treatment. Arrows indicate time of treatment: morphine (M; 0.1 mg/kg X 3), combined morphine/phenylephrine (MP; 0.1 mg/kg plus 25 µg/kg X 3) and Control (C; no treatment).

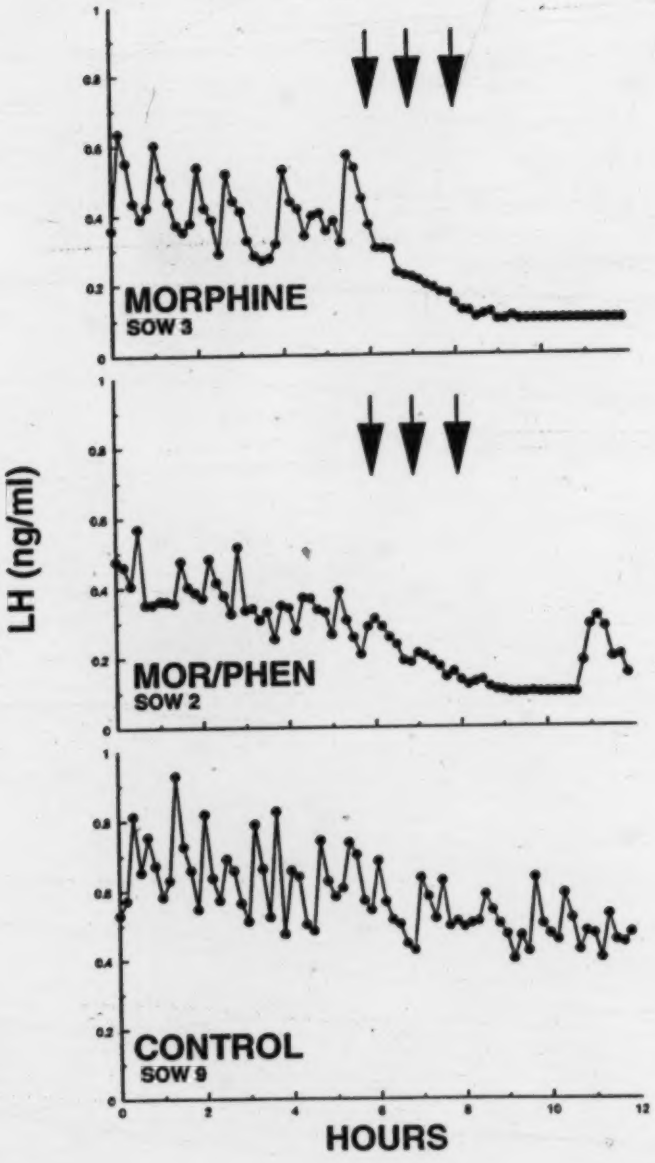

LH (ng/ml)
MORPHINE
SOW 3
MOR/PHEN
SOW 2
CONTROL
SOW 9
HOURS

TABLE 4.1 Pre- and post-treatment effect on LH pulsatility during lactation in 16 sows.

Sow	Episodic Frequency/6h					
	Naloxone		Phenylephrine		Naloxone/Phenylephrine	
	Pre-	Post-	Pre-	Post-	Pre-	Post-
1	1	1	1	0	2	2
2	0.5	3	-	-	0	1.5
4	0	0	0.5	1.5	0	0
5	0	1	1	0	1	2
6	0	2	0	0	0	1
7	1	1	1	0	0.5	1
8	0	0	0	0	0	1
9	3	5	2	2.5	6	6
10	3	4	1	1	1.5	3
11	0	2	1	0	1	0
12	1	2	2.5	3	3.5	5.5
13	0	0	1	0	0	0
14	0	0	0	0	1	0
15	0.5	3	0	0	0	2
16	1	1	1	0	1	1

TABLE 4.2 Mean (± SEM) treatment effect on LH pulsatility during lactation for 16 sows.

TREATMENT	CHANGE IN EPISODIC FREQUENCY/6H BETWEEN PRE- AND POST-TREATMENT PERIODS		
	Mean	SEM	P<
Naloxone	0.93	0.26	0.003
Phenylephrine	-0.29	0.19	0.15
Naloxone/Phenylephrine	0.57	0.25	0.04

CHAPTER 5
Noradrenergic Control of Gonadotropin Releasing Hormone (GnRH) Secretion *In Vitro* in Hypothalamic Tissue from the Cyclic Gilt

INTRODUCTION

There is ample evidence that in rats (Negro-Vilar *et al.*, 1979; Leung *et al.*, 1982; Kinoshita *et al.*, 1981; Nowak and Swerdloff, 1985; Clough *et al.*, 1988; Condon et al, 1989; Clough *et al.*, 1990; Jarry *et al.*, 1990; Martin *et al.*, 1995) and primates (Kaufman *et al.*, 1985; Terasawa *et al.*, 1988; Gearing and Terasawa, 1991) norepinephrine stimulates GnRH secretion from the hypothalamus and that these effects are mediated by α_1-adrenergic receptors. In contrast, there is a relative paucity of information on the neuroendocrine regulation of the GnRH pulse generator in the pig, although this information is fundamental to an evaluation of potential clinical approaches to enhancing fertility.

Immunohistochemical studies in the rat (Chen *et al.*, 1989; Horvath *et al.*, 1992) and the ewe (Conover *et al.*, 1993; Lehman and Karsch, 1993) have identified GnRH and catecholaminergic neurons in close apposition within the hypothalamus. Similar studies in the pig by Kineman *et al.* (1988) have identified GnRH perikarya primarily in the preoptic area of the hypothalamus and GnRH axons extending to the median eminence. In other studies Leshin *et al.* (1989 and 1996) identified tyrosine hydroxylase and dopamine-ß-hydroxylase immunopositive neurons in the same areas as GnRH neurons. These data provide an anatomical basis for interactions between noradrenergic and GnRH systems in the pig. The influence of noradrenergic stimuli on porcine GnRH is, however, still uncertain.

Early studies by Parvizi and Ellendorff (1978 and 1982) showed both stimulatory and inhibitory GnRH secretory responses when norepinephrine was administered intraventricularly or micro injected into discrete hypothalamic nuclei of Göttingen miniature pigs. More recent studies by Kesner *et al.* (1987) and Chang *et al.* (1990 and 1993) have shown that the noradrenergic synthesis inhibitors diethyldithiocarbamate (DDC) and N-methyl-N'[1-methyl-2-propenyl] 1,2 hydrazine-dicarbothioamide (AIMAX) can suppress basal and surge secretion of LH in the cyclic and ovariectomized gilt, suggesting a stimulatory noradrenergic pathway in porcine GnRH release. As steroids have a profound effect on neurotr smitter-mediated GnRH release, and in particular, estradiol facilitates the stimulatory effect of norepinephrine on GnRH neurons (Leung *et al.* 1982; Pau and Spies, 1986; Clough *et al.*, 1988; Condon *et al.*, 1989), follicular phase gilts were used in the present study. Our objective was to determine if the noradrenergic system regulates GnRH secretion *in vitro* in hypothalami, recovered from follicular phase gilts, and if so, which receptor type mediates this function.

MATERIALS AND METHODS

Animals and Tissue Collection

A total of 34 Camborough PIC gilts from the University of Alberta Swine Research

Unit were slaughtered in groups of 2 and brain tissue was collected for *in vitro* assessment of GnRH release in response to various noradrenergic receptor agonists and their respective antagonists. Gilts were shipped during the follicular phase of their cycle (d18.31 ± 1.42 (mean ± S.D.) and weighed (mean ± S.D.) 146.71 ± 21.71 kg.

Gilts were slaughtered at a commercial abattoir by electrostunning and exsanguination. Craniotomy and removal of the entire brain occurred within 10 minutes of electrostunning. A block of tissue containing the entire hypothalamic-preoptic area (HPOA) was then excised on a chilled surface, using a brain knife (see Appendix A, Fig.A.1). Cuts were made 5 mm rostral to the optic chiasm and immediately rostral to the mammillary bodies. These cuts produced a slice of tissue approximately 10 mm thick, which was then placed with the posterior surface facing up. HPOA explants were further isolated from this slice of tissue using a razor blade to cut laterally at the thalamic boundary (top of the third ventricle was used as a marker) and further cut sagittally at the third ventricle, producing two halves with a "tail" of cortical tissue that aided in stabilizing the tissue while producing hypothalamic slices for perfusion. Median eminence (ME) tissue was not included. Each half was sagittally sliced into 500 µm sections with a tissue slicer (Stoelting Co. Wood Dale, IL). On the basis of preliminary studies (Appendix A, Part 5), only the 4 slices immediately lateral to the third ventricle from each half were used for perfusion, combining slices 1 and 3, and 2 and 4, from each half, in separate transport vials. Tissue was transported back to the lab in these individual vials containing chilled, continuously oxygenated perfusion medium. Once in the lab, the two tissue slices from each transport vial were transferred to individual perfusion chambers, thereby creating eight chambers from the two gilts slaughtered on each occasion.

Ovaries from the slaughtered gilts were also collected and examined for presence of follicles and corpora lutea. Follicle size was measured and approximately 1 ml of follicular fluid was collected from the largest follicles of both ovaries and stored in polypropylene vials at -70°C until assayed for progesterone and estradiol-17β concentrations.

Perfusion System

Perfusion chambers (25mm SWINNEX filters; Millipore Corporation, Bedford MA) with a 2.3 ml capacity were maintained in a water bath at 37° C. Perfusion medium consisted of Delbecco's Modified Eagle's Medium and Ham's Nutrient Mixture F-12 (DME/F12; pH 7.3; 1.2 g $NaHCO_3$) with addition of 0.1% BSA, 0.1mM bacitracin and 5 mg kanamycin sulfate/l (Sigma Chemical Co., St. Louis, MO) as described by Barb *et al.* (1994), but excluding 5 mg amphotericin B from their original formulation. Medium was oxygenated (95% O_2-5% CO_2), warmed to 37° C and pumped through the tissue chambers at the rate of 100 µl/min. Fractions were collected at 5 min intervals (500 µl) into polypropylene vials, snap frozen and stored at -70° C until assayed for GnRH.

Prior to treatment, the 4 chambers containing the HPOA sections from each gilt were randomly assigned to one of four treatment groups, then allowed to stabilize for 120 min (1 hour of washout, no samples collected and 1 hour of collection, fractions 1-11). Various noradrenergic treatments were administered during the collection of fractions 12-34 in subsequent experiments to investigate the involvement of different receptor types in the regulation of GnRH secretion. The non-specific noradrenergic agonist, norepinephrine (NE;

Part 1), the specific α_1-adrenergic receptor agonist, phenylephrine (PHEN; Part 2) and the ß-adrenergic receptor agonist, isoproterenol (ISO; Part 4) (Sigma Chemical Co., St. Louis, MO) were used. At the start of fraction 12 the media reservoir was changed for 3 different reservoirs containing one of the drugs at 3 different doses, plus one control reservoir containing only media. Actual drug concentrations in the reservoirs were; NE 1 X 10^{-5}, 10^{-7} and 10^{-9}M; PHEN 4.91 X 10^{-5}, 10^{-7} and 10^{-9}M; ISO 4.04 X 10^{-5}, 10^{-7} and 10^{-9} M. At the beginning of fraction 34 a 1 min depolarizing pulse of 1.38M KCl was introduced from a single reservoir, to produce a final chamber concentration of 60 mM KCl, as a means of assessing tissue viability. Responses to KCl were determined, as described in Appendix 1, Part 4, and only data from tissue considered viable using these criteria were included in further statistical analysis. All drugs and KCl were diluted in perfusion medium. Treatment medium also contained 0.03% ascorbic acid, to prevent the oxidation of catecholamines (Clough *et al.*, 1988). Because all treatments were administered as continuous perfusions at a flow rate of 100 µl/min into a chamber volume of 2.3 ml, there was also clearly an inbuilt lag period in the system before threshold concentrations for stimulation were reached and indeed, initial assessment of the response profiles to drug treatment indicated that responses occurred after fractions 20-22.

Upon completion of the agonist-only trials (Parts 1,2 and 4), GnRH profiles were subjectively assessed to determine the concentration which provided the most consistent stimulatory response, prior to attempting to antagonize this response with the appropriate adrenergic receptor antagonist. Again, prior to treatment, the 4 chambers containing the HPOA sections from each gilt were randomly assigned to one of four treatment groups, then allowed to stabilize for 60 min (1 hour of washout with perfusion media, no samples collected). However, in this series of trials, during the collection of fractions 1-11 the media reservoir contained either PHEN (4.91 X 10^{-5}M) or ISO (4.04 X 10^{-5} M), so that all 8 chambers received the agonist, to maintain active GnRH secretion. At the start of fraction 12 the media reservoir was again exchanged for 3 different reservoirs each containing a different dose of the appropriate antagonist, prazosin, an α_1-adrenergic receptor antagonist, (PRAZ; Part 3; Sigma) or propranolol, a ß-adrenergic receptor antagonist (PROP; Part 5; Sigma), plus the respective agonist, and one control reservoir containing only the agonist, at the dose previously mentioned. Actual drug concentrations in the reservoirs were; PRAZ 2.38 X 10^{-5}, 10^{-7} and 10^{-9} M or PROP 3.38 X 10^{-5}, 10^{-7} and 10^{-9} M. At the beginning of fraction 34, KCl was given as previously described.

Hormone Assays

GnRH acetate salt (Sigma) was iodinated using the method of Nett and Adams (1977). GnRH was quantified in media samples using the single antibody radioimmuno assay described by Sesti and Britt (1993) and antibody kindly supplied by Dr. J.H. Britt (North Carolina State University, Raleigh, NC), with the minor modification as follows. 200 µl of media sample was added to each unknown tube, omitting 100 µl of assay buffer, so that total tube volume remained the same. The intra- and inter-assay coefficients of variation (CV) were 17.23% and 21.3%, respectively. The overall sensitivity of the assays, defined as 2 standard deviations below the B_{max} value, was 0.57 pg/tube.

Progesterone in 100 µl of follicular fluid was assayed without extraction using Coat-a-

Count Progesterone kits (Diagnostic Products Corp., Los Angeles, CA) validated for use with pig follicular fluid (Willis, 1997). Samples for the assay were diluted to either 1:100 or 1:300 in a zero calibrator. Intra- and inter-assay CVs were 2.03% and 10.31%, respectively. The assay sensitivity was 0.009 ng/tube.

Estradiol was determined in 100 µl follicular fluid using the direct method previously described by Ding and Foxcroft (1993). Samples for this assay were diluted to either 1:500 or 1:1000 in assay buffer. The intra-assay CV was 11.79% and the sensitivity was 3.13 pg/tube.

Statistical Analysis

Initial appraisal of GnRH profiles determined that a baseline (nadir) level of GnRH release was established within 100 min of perfusion (fraction 20). The mean GnRH concentration in fractions 20-22 was therefore used as a pretreatment baseline, with which relative changes in GnRH responses to adrenergic stimuli could be compared. Mean GnRH responses in sequential 15 min intervals were determined for 60 min after adrenergic stimulation (fractions 23-34). Data were expressed as percent change from baseline to reduce the effect of variation between chambers in absolute GnRH concentrations.

As previously mentioned, for Parts 3 and 5 of the study, the α- and ß-adrenergic agonists PHEN and ISO were introduced into the system at the start of fraction 1. When the GnRH profiles for each respective agonist were appraised it was clear that by the end of 1 hour (fraction 12) GnRH secretion was sufficiently maintained to allow the appropriate antagonist (PRAZ or PROP) to reduce GnRH secretion. Mean GnRH responses in sequential 15 min intervals were determined for 105 min after antagonist treatment. The mean of fractions 11-13 was used as a baseline, against which to compare changes in GnRH responses to adrenergic receptor antagonism. Data were expressed as percent change from baseline to reduce the effect of variation between chambers in absolute GnRH concentrations.

ANOVA for repeated measures (PROC GLM, SAS statistical package, 1988) was applied to the data. When overall treatment ("time") effects were significant, linear contrasts were used to compare responses over specific 15 min time periods with the baseline.

RESULTS

Overall, GnRH secretion *in vitro* was very variable between animals. Basal GnRH secretion rate (based on the mean (± S.D.) for fraction 20-22 prior to conversion to percentages, from agonist only trials) was determined to be 0.40 ± 0.41 pg/chamber/min. Data of the ovarian status of gilts at slaughter, including estradiol to progesterone ratios in pooled follicular fluid for the various drug treatment trials are listed in Table 5.1. Ascorbic acid media alone during the treatment period had no effect on GnRH secretion in any of the trials.

GnRH Response to Norepinephrine

Part 1: GnRH secretion in this group of gilts was higher and more variable than expected. There was no effect of NE treatment at 10^{-7}M and 10^{-9}M. The response to NE at

10^{-5}M approached significance (P=0.076) (Fig.5.1). The follicular data in Table 5.1 and Figure 5.2 indicate that most gilts in this group were slaughtered in the late follicular phase, and in several cases, in the post-LH surge period.

GnRH Response to α_1-Adrenergic Treatments

Part 2: There was a positive response to both PHEN at 4.91 X 10^{-5} (P=0.0004) and 10^{-9}M (P=0.0018). Phenylephrine at a dose of 4.91 X 10^{-7} M failed to stimulate GnRH secretion (P=0.27) (Fig.5.3). We have no explanation for this failure, other than variability between chambers preventing the determination of a response. Linear contrasts showed that at the highest dose, PHEN stimulated GnRH secretion at all four 15 min time points after the baseline (P≤0.01). At the lowest dose, PHEN stimulation was slightly delayed and only the last three 15 min fractions were significantly higher than baseline (P≤0.04) (Fig.5.3).

Part 3: PHEN administered at 4.91 X 10^{-5}M during collection of fractions 1-34, maintained GnRH secretion. PRAZ administered to chambers being stimulated with PHEN, at doses of 2.38 X 10^{-7}M and 10^{-9}M significantly suppressed GnRH secretion (P=0.01 and P=0.02, respectively). Contrasts made between response fractions and baseline indicated that in the 10^{-7}M group, PRAZ inhibited GnRH secretion in all but one fraction (Fig.5.4). Contrasts in the 10^{-9}M group indicated that PRAZ was unable to maintain inhibition of GnRH secretion throughout the entire treatment response period (Fig.5.4). PRAZ at a dose of 2.38 X 10^{-5}M did not significantly suppress agonist stimulated GnRH secretion, however there was a trend for GnRH secretion to decrease (P=0.10). Prazosin's failure to inhibit agonist stimulated GnRH secretion at the highest dose was likely due to high variability between chambers, similar to that seen in the PHEN 4.91 X 10^{-7}M dose.

As a point of interest, we used fraction 20-22 as the baseline in Part 3 of this study (although for statistical purposes fraction 11-13 was the actual baseline; see explanation in Materials and Methods and Appendix A, Part 6) and then compared the resulting response to the PHEN 4.91 X 10^{-5}M treatment group in Part 2 of this study. It appeared that administering PHEN an hour earlier (starting at fraction 1 in Part 3) had indeed changed the pattern of GnRH secretion seen when PHEN was administered beginning at fraction 12 (Part 2). GnRH secretion was maintained (Part 3) throughout a period in which GnRH secretion declined (Part 2), confirming the statistical results which showed that prazosin was able to antagonize stimulated GnRH secretion.

GnRH Response to ß-Adrenergic Treatments

Part 4: There was no overall response to treatment with ISO at the highest dose (P=0.13). Quite unexpectedly, there was a significant effect of "time" in both the ISO 4.04 X 10^{-7}M and 10^{-9}M treatment groups (P≤0.05). Contrasts determined that in the ISO 4.04 X 10^{-7}M group only the last time point in the treatment period was significantly different than the baseline (P=0.04) (Fig.5.5). However, all time points in the lowest ISO dosage group were significantly different from the baseline (P≤0.03) (Fig.5.5).

Part 5: Compared to PHEN, ISO at 4.04 X 10^{-9}M administered continuously during collection of fractions 1-34, failed to maintain GnRH secretion (Fig.5.6). PROP failed to inhibit agonist induced GnRH secretion at any of the three doses used (Fig.5.6).

DISCUSSION

Neural regulation of GnRH release in the pig is still uncertain and there is a paucity of information on GnRH release from the porcine hypothalamus *in vitro*. However, the results of the present study strongly indicate the participation of an adrenergic system in the regulation of GnRH release and that an *in vitro* model for subsequent trials is a viable alternative to traditional *in vivo* models.

Mean GnRH secretion rates from hypothalamic explants in this study are lower than those reported by Barb *et al.* (1994), likely due to their inclusion of entire, sectioned hypothalamic halves in their perfusion chambers. However, they are higher than mean GnRH secretion rates reported by Cox *et al.* (1990) from ovariectomized gilts, where whole halves of hypothalami were perfused for 4 hours. Another study conducted by Sesti and Britt (1993) using a static incubation of POA/suprachiasmatic and MBH explants could not detect GnRH levels in the incubation media. This may have been due to poor diffusion of GnRH out of tissues, similar to the Cox study, or to very low GnRH secretion rates in hypothalamic tissue obtained from early weaned and lactating sows. Although other studies in the pig have implicated the involvement of noradrenergic mechanisms in the regulation of GnRH, and thus LH, secretion (Parvizi and Ellendorff, 1978 and 1982; Leshin *et al.*, 1989 and 1996; Chang *et al.*, 1990 and 1993) this is the first study to investigate specific receptor types involved in noradrenergic regulation of GnRH secretion and to demonstrate an α_1-receptor mediated stimulation of GnRH secretion from the porcine hypothalamus *in vitro*.

Initially, we sought to demonstrate that NE, a mixed α- and β-receptor agonist (Weiner, 1980), regulates GnRH secretion from the hypothalamus. However, we were unable to demonstrate a clear stimulatory or inhibitory response at any dosage of the drug. Unequivocal results have previously been reported in many studies using rats, in which the outcome was dependent on the age, physiological state and steroidal environment of the test animal, and upon the route of administration and dosage of NE used (Klieg *et al.*, 1976; Negro Vilar *et al.*, 1979; Leung *et al.*, 1981 and 1982; Nowak and Swerdloff, 1985). Parvizi and Ellendorff (1978) found that NE (10^{-4}M) given intraventricularly elevated plasma LH in male miniature pigs but when applied directly into periventricular structures, NE significantly inhibited plasma LH. In a later experiment, Parvizi and Ellendorff (1982) administered various doses of NE into discrete hypothalamic regions of ovariectomized miniature pigs. Higher doses of NE in the dorsal area of the hypothalamus inhibited, while lower doses stimulated, LH secretion. The converse was true in the dorsomedial nucleus. There were no effects on LH secretion when NE was microinjected into the ventromedial nucleus.

There are four possible reasons for the lack of a response to the three different doses of NE. 1) the catecholamine degraded in the culture media over the 110 min treatment period. It has been reported that the biological activity of NE in culture media (Medium 199) is reduced by 50% after 1 hour (Pau and Spies, 1986); although this is unlikely to have affected our results since ascorbic acid was added to the media prior to NE, to stabilize the catecholamine. 2) the dosages were insufficient to elicit a response. However, this is also unlikely, since Parvizi and Ellendorff (1982) used similar dosages which either inhibited, stimulated or did not affect GnRH secretion, depending on the hypothalamic site of injection.

3) when the day of cycle was calculated for these animals it was found to be later in the follicular phase than for the other drug trials combined ((mean ± S.D.) d20.25 ± 0.89 vs d17.67 ± 0.87). Indeed, despite the fact that large preovulatory size follicles were present on the ovary, half of these animals had declining follicular fluid estradiol concentrations and elevated progesterone concentrations, and the estrogen to progesterone ratio was less than 1 in these cases (Table 5.1). This suggests that these animals had luteinizing follicles and had already been exposed to the LH surge (Grant, 1989). It is therefore possible that the high pre-surge secretion of GnRH associated with this timing may have masked any treatment effects of NE. We therefore attempted to slaughter the remaining animals in the other trials earlier in their follicular phase. Nevertheless, the most likely explanation for the lack of a GnRH response to NE was 4) the cumulative effects of NE, a known α- and β-receptor agonist (Weiner, 1980), on the hypothalamic tissue *in vitro*. Both receptor types have been shown to affect GnRH secretion: α being stimulatory and β tending to be inhibitory (Cáceres and Taleisnik, 1980 and 1982; Dotti and Taleisnik, 1984; reviewed by Taleisnik and Sawyer, 1986). Perfusion of the tissue in this system allowed NE access to several different nuclei and neuronal systems, many of which may impinge on the GnRH system, and in effect, the stimulatory and inhibitory actions of this non-specific agonist may have cancelled one another out.

In contrast to NE, phenylephrine, demonstrated that the α_1-noradrenergic system is involved in regulation of GnRH secretion in the porcine hypothalamus. Phenylephrine stimulated GnRH secretion and this effect was reversible by the specific α_1-receptor antagonist, prazosin. Again, several reports of α_1-receptor mediated stimulation of GnRH secretion in the hypothalamus exist for the rat (Negro-Vilar *et al.*, 1979; Leung *et al.*, 1982; Kinoshita *et al.*, 1981; Nowak and Swerdloff, 1985; Condon et al, 1989; Clough *et al.*, 1990; Jarry *et al.*, 1990; Leonhardt *et al.*, 1991) and primate (Kaufman *et al.*, 1985; Terasawa *et al.*, 1988; Gearing and Terasawa, 1991), but this is the first time that a specific receptor type has been demonstrated to mediate stimulatory or inhibitory actions on GnRH secretion in the gilt.

Unexpectedly, we saw a significant stimulation of GnRH secretion using the β-agonist, isoproterenol, at a dose of 4.04×10^{-9}M in the agonist only trial (Part 4). However, this effect could not be repeated or reversed in the combined trial (Part 5), using the specific β-antagonist, propranolol, at any dose. It is therefore possible that this effect was artifactual and does not represent actual stimulation. Similar effects of dose have been reported for some dopaminergic drugs, lower doses being more stimulatory to both dopamine and prolactin secretion than higher doses (Burris *et al.*, 1991; Tagawa *et al.*, 1992; Damsma *et al.*, 1993; Depoortere *et al.*, 1996; Schoemaker *et al.*, 1997).

Studies have reported that β-receptors may be responsible for mediating inhibitory actions on the GnRH pulse generator (Cáceres and Taleisnik, 1980 and 1982; Dotti and Taleisnik, 1984; reviewed by Taleisnik and Sawyer, 1986; Condon *et al.*, 1989) and that many of these inhibitory actions on GnRH occur in the absence of steroids, particularly estradiol. Other studies have reported no effect of β-adrenergic agents on GnRH secretion (Nowak and Swerdloff, 1985; Gearing and Terasawa, 1991; Leonhardt *et al.*, 1991). Inhibitory actions of the noradrenergic system on GnRH neurons may also be mediated by interneurons, such as GABA, a known inhibitory neurotransmitter, interacting with noradrenergic inputs to affect

the GnRH pulse generator.

Anatomical studies in rats have demonstrated GABA neuronal interactions with GnRH neurons (Jennes *et al.*, 1983; Leranth *et al.*, 1985). Several studies, using both *in vivo* and *in vitro* techniques, have shown that the GABA system interacts with the noradrenergic system to regulate GnRH secretion when estradiol is present. However, there are two lines of reasoning; 1) that noradrenaline stimulates GABA secretion, which in turn inhibits GnRH secretion (Herbison *et al.*, 1989 and 1990), and 2) that GABA inhibits noradrenergic neurons which would normally stimulate GnRH secretion (Adler and Crowley, 1986; Akema *et al.*, 1990; Brann *et al.*, 1992; Akema and Kimura, 1993).

This is the first study to demonstrate *in vitro* a stimulatory α_1-adrenoreceptor mediated action on GnRH secretion from the hypothalamus of the gilt. It appears that during the follicular phase, a noradrenergic input to the GnRH neuron exists. However, it remains to be elucidated what other inputs impinge on this system, particularly with respect to different physiological paradigms. With the lack of good adrenergic pharmaceuticals that will cross the blood brain barrier without causing peripheral effects, the study of GnRH secretion from the hypothalamus *in vitro* is a viable alternative to traditional methodologies. This technique has been a useful tool to help elucidate the function of the GnRH pulse generator in many other species, and can now successfully be used in the pig.

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FIGURE 5.1 15 minute mean (+ or - SEM) GnRH concentration in fractions collected during *in vitro* perfusion of hypothalamic tissue from 8 follicular phase gilts, expressed as a percentage change from baseline (fraction 20-22, not shown). 15 minute fractions 23-34 represent the GnRH secretory responses to treatment with ascorbic acid media, NE 1 x 10^{-5}, 10^{-7} and 10^{9} M (see legend). See figure for the number of chambers represented in each treatment group.

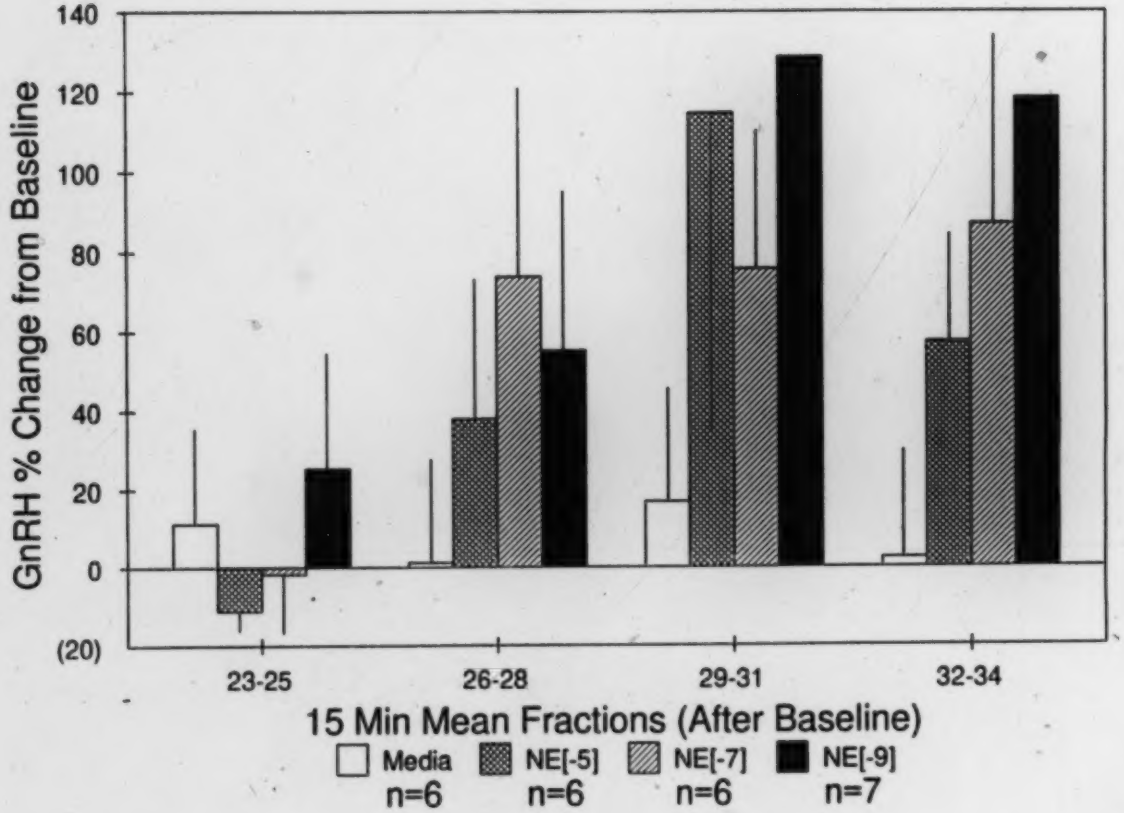
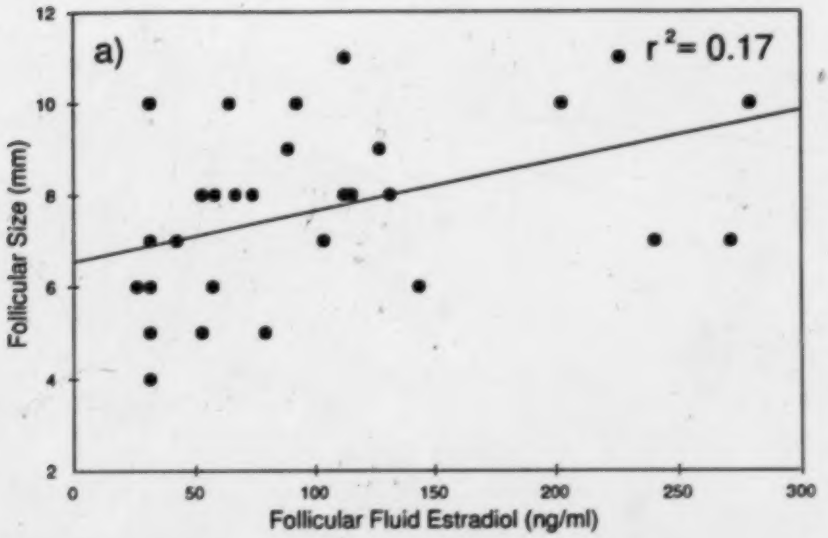

140
120
100
80
60
40
20
0
(20)
GnRH % Change from Baseline
23-25
26-28
29-31
32-34
15 Min Mean Fractions (After Baseline)
Media
NE[-5]
NE[-7]
NE[-9]
n=6
n=6
n=6
n=7

FIGURE 5.2 The correlation between a) follicular size and estradiol concentration and b) estradiol and progesterone concentrations, in pooled follicular fluid based on data from 32 gilts. r^2 for the entire follicular population was 0.06. However, when the post LH surge follicles (solid squares) and pre-ovulatory population (solid triangles) were omitted form the analysis, r^2 was equal to 0.41 for the population of growing estrogenic follicles (solid circles).


a)
$r^2 = 0.17$
Follicular Size (mm)
Follicular Fluid Estradiol (ng/ml)
12
10
8
6
4
2
0
50
100
150
200
250
300

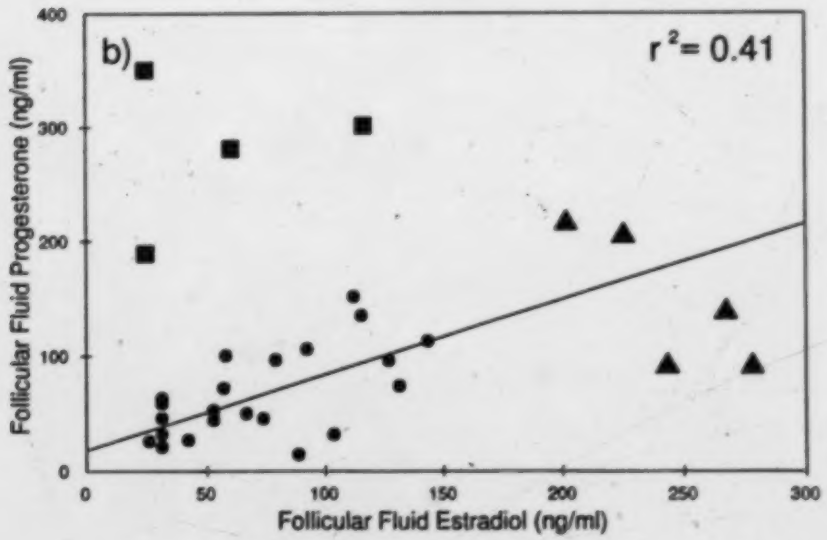

b)
$r^2 = 0.41$
Follicular Fluid Progesterone (ng/ml)
Follicular Fluid Estradiol (ng/ml)
400
300
200
100
0
50
100
150
200
250
300

FIGURE 5.3 15 minute mean (+ or - SEM) GnRH concentration in fractions collected during *in vitro* perfusion of hypothalamic tissue from 6 follicular phase gilts, expressed as a percentage change from baseline (fraction 20-22, not shown). 15 minute fractions 23-34 represent the GnRH secretory responses to treatment with ascorbic acid media, PHEN (α_1-adrenergic agonist) 4.91 X 10^{-5}M, ** denotes means significantly differ from fraction 20-22 ($P \leq 0.01$), PHEN 4.91 X 10^{-7}M, and PHEN 4.91 X 10^{9} M, * denotes means significantly differ from fraction 20-22 ($P \leq 0.04$). See figure for the number of chambers represented in each treatment group.

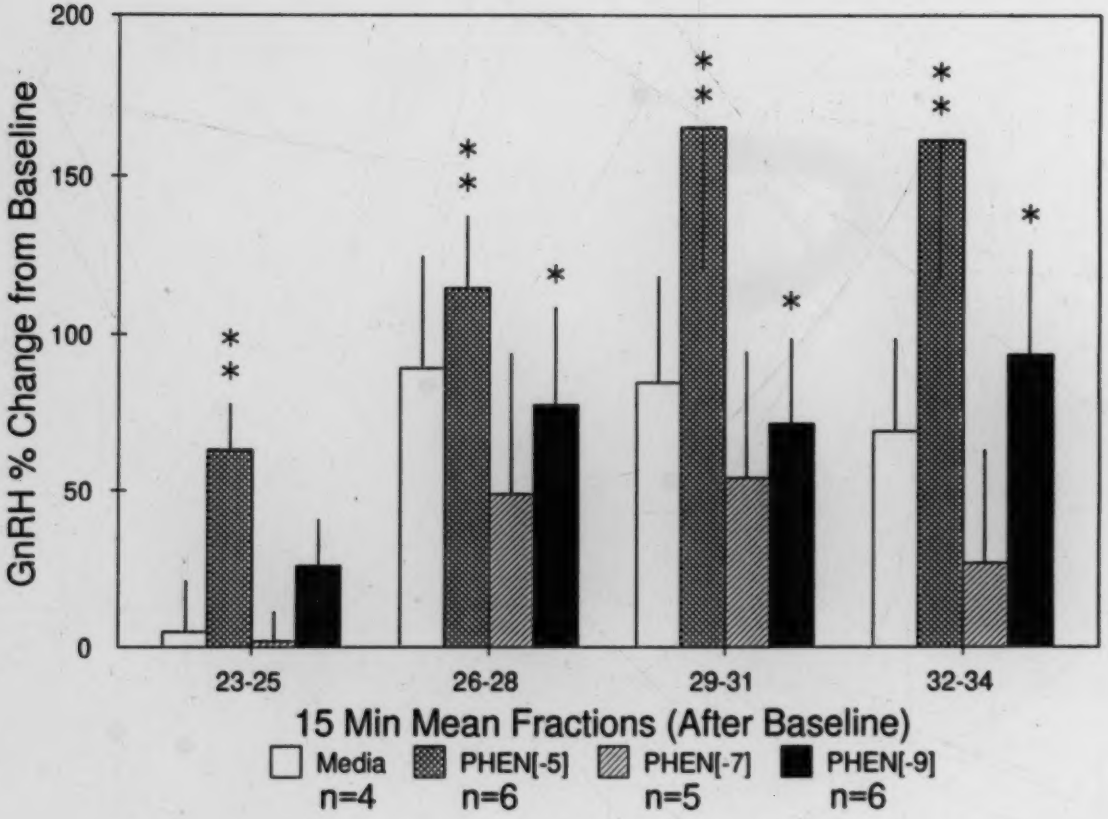

GnRH % Change from Baseline
200
150
100
50
0
23-25
26-28
29-31
32-34
15 Min Mean Fractions (After Baseline)
Media
PHEN[-5]
PHEN[-7]
PHEN[-9]
n=4
n=6
n=5
n=6

FIGURE 5.4 15 minute mean (+ or - SEM) GnRH concentration in fractions collected during *in vitro* perfusion of hypothalamic tissue from 6 follicular phase gilts, expressed as a percentage change from baseline (fraction 11-13, not shown). 15 minute fractions 14-34 represent the GnRH secretory responses to treatment with PHEN (α_1-adrenergic agonist) 4.91 X 10^{-5}M administered continuously throughout the perfusion, PHEN 4.91 X 10^{-5}M and PRAZ (α_1-adrenergic antagonist) 2.38 X 10^{-5}M combined, PHEN 4.91 X 10^{-5}M and PRAZ 2.38 X 10^{-7}M combined, * denotes means significantly differ from fraction 11-13 ($P \leq 0.05$), and PHEN 4.91 X 10^{-5}M and PRAZ 2.38 X 10^{-9}M combined, ** denotes means significantly differ from fraction 11-13 ($P \leq 0.01$). See figure for the number of chambers represented in each treatment group.

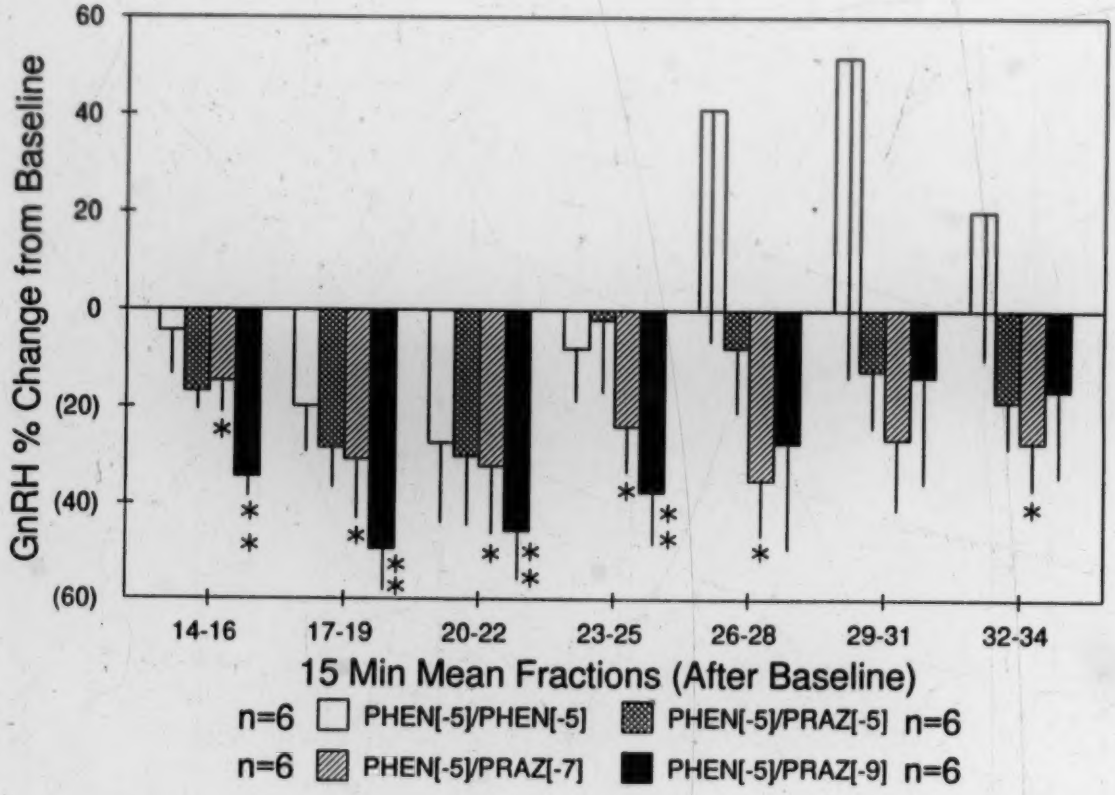

60
40
20
0
(20)
(40)
(60)
GnRH % Change from Baseline
14-16
17-19
20-22
23-25
26-28
29-31
32-34
15 Min Mean Fractions (After Baseline)
n=6
PHEN[-5]/PHEN[-5]
PHEN[-5]/PRAZ[-5]
n=6
n=6
PHEN[-5]/PRAZ[-7]
PHEN[-5]/PRAZ[-9]
n=6

FIGURE 5.5 15 minute mean (+ or - SEM) GnRH concentration in fractions collected during *in vitro* perfusion of hypothalamic tissue from 8 follicular phase gilts, expressed as a percentage change from baseline (fraction 20-22, not shown). 15 minute fractions 23-34 represent the GnRH secretory responses to treatment with ascorbic acid media, ISO (β-adrenergic agonist) 4.04 X 10^{-5}M, ISO 4.04 X 10^{-7}M, * denotes means significantly differ from fraction 20-22 ($P \leq 0.04$), and ISO 4.04 X 10^{-9}M, * denotes means significantly differ from fraction 20-22 ($P \leq 0.03$). See figure for the number of chambers represented in each treatment group.

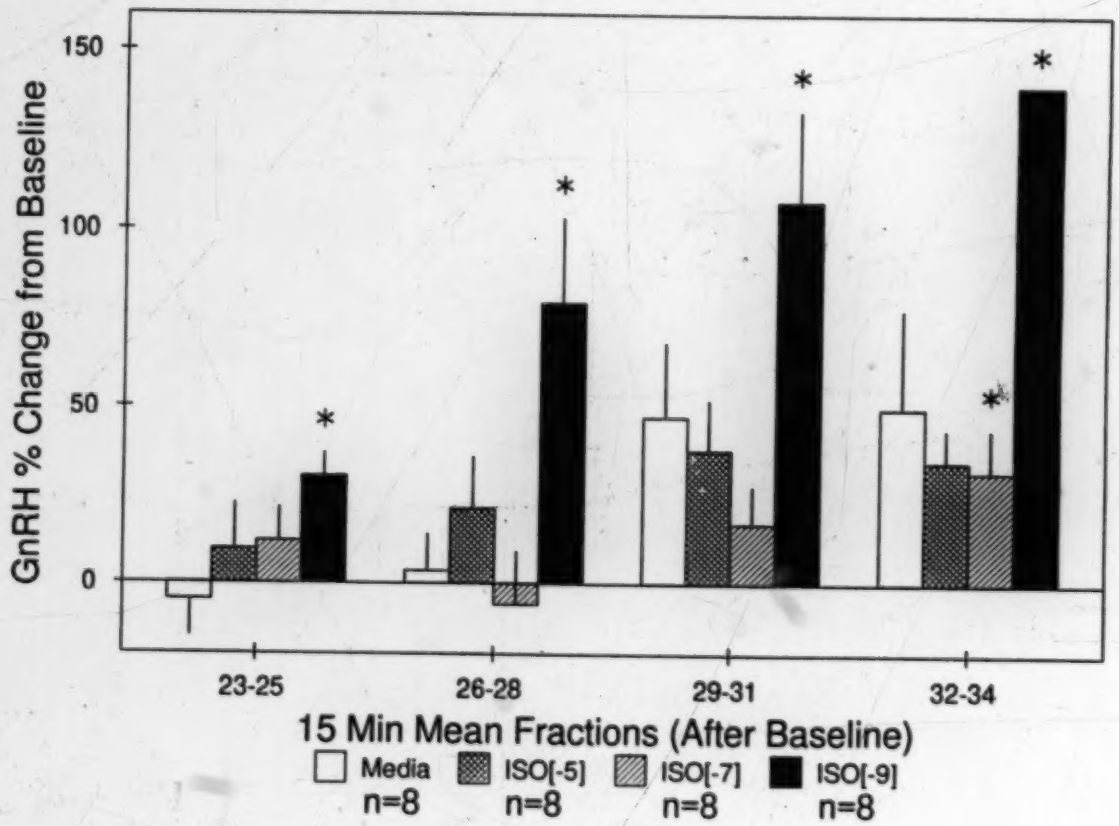

150
100
50
0
GnRH % Change from Baseline
*
*
*
*
23-25
26-28
29-31
32-34
15 Min Mean Fractions (After Baseline)
Media
n=8
ISO[-5]
n=8
ISO[-7]
n=8
ISO[-9]
n=8

FIGURE 5.6 15 minute mean (+ or - SEM) GnRH concentration in fractions collected during *in vitro* perfusion of hypothalamic tissue from 6 follicular phase gilts, expressed as a percentage change from baseline (fraction 11-13, not shown). 15 minute fractions 14-34 represent the GnRH secretory responses to treatment with to ISO (β-adrenergic agonist) 4.04 X 10^{-9}M administered continuously throughout the perfusion, ISO 4.04 X 10^{-9}M and PROP (β-adrenergic antagonist) 3.38 X 10^{-5}M combined, ISO 4.04 X 10^{-9}M and PROP 3.38 X 10^{-7}M combined, and ISO 4.04 X 10^{-9}M and PROP 3.38 X 10^{-9}M combined. See figure for the number of chambers represented in each treatment group.

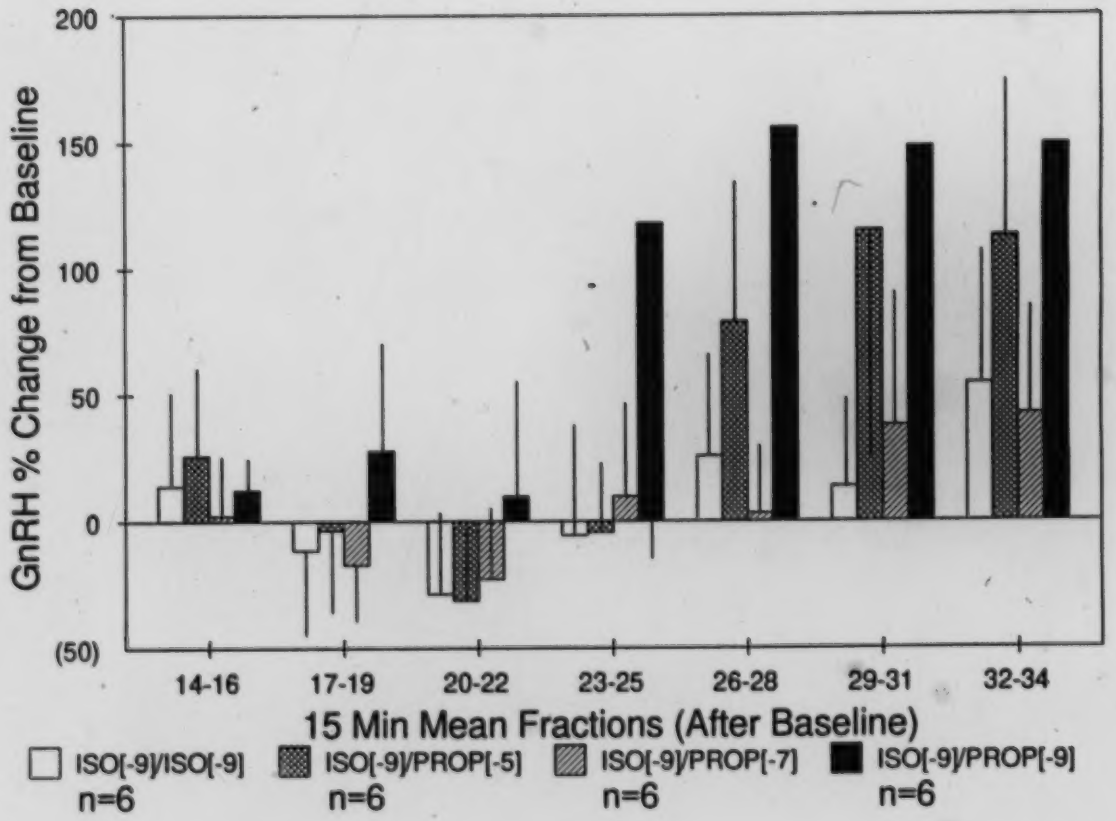

200
150
100
50
0
(50)
GnRH % Change from Baseline
14-16
17-19
20-22
23-25
26-28
29-31
32-34
15 Min Mean Fractions (After Baseline)
ISO[-9]/ISO[-9]
n=6
ISO[-9]/PROP[-5]
n=6
ISO[-9]/PROP[-7]
n=6
ISO[-9]/PROP[-9]
n=6

TABLE 5.1 Shipping data for gilts in Parts 1-5 of the experiment. Mean (± S.D.) weight = 146.71 ± 21.71 kg; Mean (± S.D.) day of cycle (for all drug trials) = 18.31 ± 1.42; Mean (± S.D.) day of cycle (NE trial) = 20.25 ± 0.89; Mean (± S.D.) day of cycle (PHEN, PRAZ, ISO and PROP trials) = 17.67 ± 0.87. The last 3 columns contain data on follicular steroid concentrations in pooled follicular fluid from each of 32 gilts. For the basis of the regression analysis in Figure 5.2, follicular sizes in trials NE3 and NE4 (for 4 gilts) were allocated as 10 mm, as they were only subjectively described as "large" at the time of study.

Trial (rep)	Shipped	Gilt	Weight	Cycle Day	Follicle Size (mm)	Progesterone (ng/ml)	Estradiol (ng/ml)	Estradiol : Progesterone
NE(1)	May 13	151	167	19	large			
NE(1)	May 13	127	175	21	medium			
NE(2)	May 15	134	176	19	6-11	204.69	226.25	1.11
NE(2)	May 15	117	163	20	6-11	289.53	112.45	0.39
NE(3)	May 22	174	133	21	large (10)	190.38	31.25	0.16
NE(3)	May 22	141	195	20	large (10)	99.81	279.13	2.80
NE(4)	May 27	139	188	21	large (10)	105.63	92.34	0.87
NE(4)	May 27	180	161	21	large (10)	353.97	31.15	0.09
PHEN(1)	May 29	176	169	17	3-5	21.13	31.25	1.48
PHEN(1)	May 29	220	138	17	8-9	14.61	88.86	6.08
PHEN(3)	June 5	212	135	18	8-10	273.33	64.00	0.23
PHEN(3)	June 5	193	150	18	6-8/9	100.11	57.93	0.58
PHEN(4)	June 24	351	152	17	4-6	45.94	31.25	0.68
PHEN(4)	June 24	360	128	17	7-8	73.68	131.56	1.79
ISO(1)	June 10	197	138	18	5-6	25.94	25.93	1.00
ISO(1)	June 10	217	152	18	6-8	52.59	52.75	1.00
ISO(2)	June 12	248	116	19	6-7	31.86	103.60	3.25
ISO(2)	June 12	195	147	18	3-5	32.16	31.25	0.97
ISO(3)	June 17	211	175	17	6-8	151.47	112.17	0.74
ISO(3)	June 17	203	178		5-7	26.92	42.23	1.57
ISO(4)	July 2	353	140	18	7-9	96.03	127.11	1.32
ISO(4)	July 2	236	115	18	4-7	21.30	31.25	1.47
PHEN/PRAZ(1)	Aug 19	379	129	18	7-8	45.50	73.76	1.62
PHEN/PRAZ(1)	Aug 19	384	145	19	8-10	213.24	202.66	0.95
PHEN/PRAZ(2)	Aug 21	371	120	17	4-5	44.42	52.64	1.19
PHEN/PRAZ(2)	Aug 21	372	128	19	5-6 (8)	112.70	143.49	1.27
PHEN/PRAZ(3)	Aug 26	355	136	18	7-8	134.78	115.47	0.86
PHEN/PRAZ(3)	Aug 26	247	141	19	5-7	100.84	240.54	2.39
ISO/PROP(1)	Sep 25	303	123		7-8	49.91	66.60	1.33
ISO/PROP(1)	Sep 25	318	116	16	7	131.26	271.29	2.07
ISO/PROP(2)	Sep 30	304	140	18	5	96.52	79.16	0.82
ISO/PROP(2)	Sep 30	388	150	16	3-4	63.14	31.25	0.50
ISO/PROP(3)	Oct 2	315	120	17	2-4	58.60	31.25	0.53
ISO/PROP(3)	Oct 2	386	149	17	5-6	72.19	57.04	0.79

CHAPTER 6
Noradrenergic/Opioidergic Regulation of Gonadotropin Releasing Hormone (GnRH) Secretion *In Vitro* in Hypothalamic Tissue from the Pregnant Sow

INTRODUCTION

There is abundant data from several species that norepinephrine (NE) stimulates GnRH secretion from the hypothalamus and that these effects are mediated by α_1 adrenoreceptors (Kinoshita *et al.*, 1981; Leung *et al.*, 1982; Kaufman *et al.*, 1985; Nowak and Swerdloff, 1985; Terasawa *et al.*, 1988; Jarry *et al.*, 1990; Gearing and Terasawa, 1991; Martin *et al.*, 1995). Evidence also shows that endogenous opioid peptides (EOPs) act at the level of the noradrenergic neurons, presynaptically, to inhibit GnRH, and therefore, LH secretion (Miller *et al.*, 1985; Diez-Guerra *et al.*, 1987; Dyer and Grossmann, 1988; Clough *et al.*, 1990; Dyer *et al.*, 1991; Nishihara *et al.*, 1991).

In the pig there is a general lack of information regarding the neural regulation of the GnRH and LH pulse generation. However, a number of laboratories have shown that EOPs are involved in the inhibition of LH secretion during established lactation in the sow (Barb *et al.*, 1986a; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988a and 1988b; De Rensis, 1993; De Rensis *et al.*, 1993). After weaning, this opioidergic inhibition is removed (Barb *et al.*, 1986a; Armstrong *et al.*, 1988a and 1988b) and LH secretion increases dramatically in many sows (for reviews see Edwards, 1982 and Foxcroft *et al.*, 1995). The EOP also function during the luteal phase of the estrous cycle (Barb *et al.*, 1985 and 1986b), mid-gestation (Szafranska *et al.*, 1994) and late gestation (Willis *et al.*, 1996) in the sow, when progesterone concentrations are high. During lactation the EOPs appear to act at the hypothalamic level to decrease GnRH release, as exogenous GnRH administration can overcome opioidergic inhibition of LH secretion (Barb *et al.*, 1986a; Sesti and Britt, 1993; De Rensis, 1993). Furthermore, Barb *et al.* (1994) have recently provided evidence for opioidergic modulation of GnRH release from hypothalamic preoptic area explants in the pig. Barb *et al.* (1990) have also demonstrated *in vitro* that beta-endorphin may interfere with gonadotrophin secretion at the pituitary level.

Kineman *et al.* (1988 and 1989) have identified GnRH perikarya, primarily in the preoptic area, and GnRH axons, extending to the median eminence, and proopiomelanocortin immunoreactive neurons within the same regions of the brain. A similar study identified tyrosine hydroxylase and dopamine-ß-hydoxylase immunopositive neurons in the same area as GnRH neurons (Leshin *et al.*, 1989 and 1996). These data provide an anatomical basis for interactions between opioidergic and noradrenergic systems in the regulation of GnRH secretion in the pig. Parvizi and Ellendorff (1978 and 1982) demonstrated that NE is capable of stimulating GnRH secretion from the hypothalamus of the pig, while Kesner *et al.* (1987) and Chang *et al.* (1989 and 1993) have shown that noradrenergic synthesis inhibitors can suppress LH secretion in the ovariectomized (OVX) gilt. In a recent study, it has been shown that the α_1-adrenergic agonist, phenylephrine, stimulates GnRH secretion from hypothalamic tissue *in vitro* from follicular phase gilts (Willis, 1997). Furthermore, it appears that opioid

peptides may be involved in the regulation of NE and GnRH secretion in the pig (Chang *et al.*, 1993). However, it is still uncertain whether EOPs released within the hypothalamus act at the presynaptic level, inhibiting stimulatory catecholamines, or directly on the GnRH neuron (reviewed by Foxcroft, 1992). Therefore, the objectives of this study were to 1), determine *in vitro* if the EOPs inhibit GnRH secretion from the hypothalamus in early gestation, and 2) if so, whether they exert their influence pre-synaptically, at the level of the noradrenergic neuron, or post-synaptically, directly at the level of the GnRH neuron.

MATERIALS AND METHODS

Animals and Tissue Collection

A total of 12 pregnant second parity Camborough PIC sows (first trimester, (mean ± S.D.) d29.5 ± 3.73), from the University of Alberta Swine Research Unit were slaughtered in groups of two, and brain tissue collected for *in vitro* assessment of GnRH release in response to the opioid antagonist, naloxone (NAL), and the α_1-noradrenergic receptor agonist, phenylephrine (PHEN).

Sows were slaughtered at a commercial abattoir by electro-stunning and exsanguination. Craniotomy and removal of the entire brain occurred within 10 minutes of electro-stunning. A block of tissue containing the entire hypothalamic-preoptic area (HPOA) was then excised on a chilled surface, using a brain knife. Cuts were made 5 mm rostral to the optic chiasm (at the anterior tip of the hypothalamic sulcci) and immediately rostral to the mammillary bodies. These cuts produced a slice of tissue approximately 10 mm thick, which was then placed posterior side up. HPOA explants were further isolated from the slice of tissue using a razor blade to cut laterally at the thalamic boundary (top of the third ventricle was used as a marker) and further cut sagittally at the third ventricle, producing two halves with a "tail" of cortical tissue to aid in stabilizing the tissue while producing hypothalamic slices for perfusion. Median eminence (ME) tissue was not included. Each half was then sagittally sliced into 500 µm sections with a tissue slicer (Stoelting Co. Wood Dale, IL). On the basis of preliminary studies (Appendix A, Part 5), only the 4 slices immediately lateral to the third ventricle from both halves were included in the perfusion, combining slices 1 and 3, and 2 and 4 from each half, in separate transport vials (see Appendix A, Fig.A.1). Tissue was transported back to the lab in these individual transport vials containing chilled, continuously oxygenated (5% CO_2, 95% O_2) perfusion medium. Once in the lab, the two tissue slices form each transport vial were transferred to individual perfusion chambers, thereby creating eight chambers from the two sows slaughtered on each occasion.

Perfusion System

Both sets of tissue slices from each half of each HPOA explant were placed into perfusion chambers with a 2.3 ml capacity (25mm SWINNEX filters, Millipore Corporation, Bedford MA) and maintained at 37° C. Perfusion medium consisted of Delbecco's Modified Eagle's Medium and Ham's Nutrient Mixture F-12 (DME/F12; pH 7.3; 1.2 g $NaHCO_3$) with addition of 0.1% BSA, 0.1mM bacitracin and 5 mg kanamycin sulfate/l (Sigma Chemical Co., St. Louis, MO) as described by Barb *et al.* (1994), but excluding 5 mg amphotericin B from their original formulation. Medium was oxygenated (95% O_2-5% CO_2), warmed to 37° C

and pumped through tissue chambers at the rate of 100 µl/min. Fractions were collected at 5 min intervals (500 µl) into polypropylene vials, snap frozen and stored at -70° C until assayed for GnRH.

Prior to treatment, the 4 chambers containing the HPOA sections from each sow was randomly assigned to one of four treatment groups, then allowed to stabilize for 120 min (1 hour of washout, no samples collected and 1 hour of collection, fractions 1-11). Treatment was administered during fractions 12-34; at the start of fraction 12 the media reservoir was exchanged for 3 different reservoirs containing one of the drugs at 3 different doses, plus one control reservoir containing media only. The reservoirs contained both drugs at the actual concentrations of: NAL 2.71 X 10^{-5}M, 10^{-7}M and 10^{-9}M (Part 1), and PHEN 4.91 X 10^{-5}M, 10^{-7}M and 10^{-9}M (Part 2). At the beginning of fraction 35 all explants were exposed to a 1 min pulse of 1.38M KCl, to produce a final chamber concentration of 60 mM KCl, to assess tissue viability. Responses to KCl were determined, as described in Appendix 1, Part 4, and only data from tissue considered viable using these criteria were included in further statistical analysis. All drugs were purchased from Sigma Chemical Co., St. Louis, MO, and were diluted in perfusion medium. PHEN treatment medium also contained 0.03% ascorbic acid to prevent the oxidation of the catecholamine (Clough *et al.*, 1988).

Hormone Assay

GnRH acetate salt (Sigma) was iodinated using the method of Nett and Adams (1977). GnRH was quantified in media samples using the single antibody RIA described by Sesti and Britt (1993) with the minor modifications as follows. 200 µl of media sample was added to each unknown tube, omitting 100 µl of assay buffer, so that total tube volume remained the same; and 10,000 cpm of radiolabelled GnRH were added to each tube and counted for 5 min. Antibody was kindly supplied by Dr. J.H. Britt (North Carolina State University, Raleigh, NC). The intra- and inter-assay coefficients of variation were 18.01% and 21.23%, respectively. The overall sensitivity of the assays was 0.472 pg/tube.

Statistical Analysis

Because all treatments were administered as continuous perfusions at a flow rate of 100 µl/min into a chamber volume of 2.3 ml, there was clearly an inbuilt lag period in the system before threshold concentrations for stimulation of GnRH secretion would be reached. Initial appraisal of GnRH profiles determined that a baseline (nadir) level of GnRH release was established within 100 min of perfusion (fraction 20), and that GnRH responses to drug treatments occurred after this time. The mean GnRH concentration in fractions 20-22 was therefore used as a pretreatment baseline, with which relative changes in GnRH responses to opioidergic antagonism or adrenergic stimulation could be compared. Mean GnRH responses in sequential 15 min intervals were determined for 60 min after the baseline (fractions 23-34). Data were expressed as percent change from baseline to reduce the effect of variation between chambers in absolute GnRH concentrations. ANOVA for repeated measures (PROC GLM, SAS statistical package, 1988) was applied to the data, using "time" as the main effect and variation within "time" as the error term, where "time" is the mean GnRH response (percent change from baseline) at one of the 4 sequential 15 min intervals following the baseline. This analysis includes the baseline fraction. When there was an overall significant

effect of "time", linear contrasts were made between each of the 4 treatment response fractions and the baseline and a difference between baseline and any subsequent 15 min mean was taken as a true response to treatment.

RESULTS

Overall, GnRH secretion *in vitro* was very variable between sows. Basal GnRH secretion rate (based on the mean (± S.D.) for fraction 20-22 prior to conversion to percentages) was determined to be 0.43 ± 0.32 pg/chamber/min. Neither media or ascorbic acid media had any effect on GnRH secretion (Figs.1 and 3).

Part 1: GnRH Response to Naloxone

There was a significant effect of "time" ($P=0.001$) for NAL at a dose of 2.71×10^{-5}M, allowing the more important analysis of linear contrasts to be made. Linear contrasts determined that at this dose, NAL significantly ($P \leq 0.05$) stimulated GnRH secretion from HPOA explants *in vitro* at all remaining time points (Fig.6.1). There was no overall effect of NAL at the doses of 2.71×10^{-7}M and 10^{-9}M ($P \geq 0.09$) (Fig.6.1). Figure 6.2 (a and b) shows GnRH profiles from two separate perfusions to demonstrate the variability between sows (different baselines) but that overall, there is a consistent increase in GnRH secretion in response to NAL at 2.71×10^{-5}M.

Part 2: GnRH Response to Phenylephrine

There was no significant GnRH response ($P=0.21$) to PHEN at 4.91×10^{-5}M (Fig.6.3). However, at 4.91×10^{-7}M and 4.91×10^{-9}M, overall responses to "time" were significant ($P=0.04$). Linear contrasts made in both of these groups showed that there were no significant ($P \geq 0.11$) contrasts between the baseline and any of the 4 subsequent 15 min fractions following the baseline (fractions 23-34) (Fig.6.3). These results indicate that the GnRH secretion rate at any of these time points was not significantly different than baseline GnRH secretion. Figure 6.4 (a and b) shows GnRH profiles from two separate perfusions to demonstrate the variability between sows; the baselines are similar but the response to treatment with PHEN 4.91×10^{-5}M varies between animals.

DISCUSSION

Mean GnRH secretion rates from hypothalamic explants in this study are similar to those recorded in a study by Willis (1997) using follicular phase gilts, although the variation between chambers is lower in this study using tissue obtained from pregnant sows. However, GnRH secretion rates are lower than those reported by Barb *et al.* (1994), using tissue from follicular phase gilts, OVX steroid replaced gilts and OVX controls, indicating that the physiological state of the animal from which tissue is obtained may be important. Differences may also be due to their use of entire sectioned hypothalamic halves in their perfusion system, compared to only two tissue slices adjacent to the third ventricle being included in each of our chambers. Barb *et al.* (1994) have shown *in vitro* that the opioid antagonist, naloxone, at a

dose similar to that used in this study, was capable of stimulating GnRH secretion from hypothalamic tissue from gilts, irrespective of the steroidal milieu, and that this effect was reversible in the presence of the opioid agonist, morphine. In the current study, naloxone was used to stimulate GnRH secretion from hypothalamic tissue, demonstrating, for the first time, an opioidergic inhibition of GnRH secretion in the early pregnant sow *in vitro*.

Several studies have demonstrated an *in vivo* EOP inhibition of GnRH secretion in a progesterone dominated environment (see reviews by: Barb *et al.*, 1991; Okrasa *et al.*, 1992). Naloxone increased LH secretion in luteal phase and mature OVX-progesterone treated gilts (Barb *et al.*, 1985, 1986 and 1988) but not in early or late follicular phase, prepubertal or OVX gilts (Barb *et al.*, 1986 and 1988). Estienne *et al.* (1990) showed that morphine administered in to the lateral ventricle inhibits LH secretion in OVX gilts, although these animals did not undergo steroid replacement therapy. Studies during gestation in the sow, another physiological paradigm in which progesterone dominates, have also shown naloxone to be effective in stimulating LH secretion on day 40 (Szafranska *et al.*, 1994) and as late as day 108 (Willis *et al.*, 1996). As well as data from the previous two studies supporting an opioidergic inhibition of LH during gestation, studies in rats show an increase in μ-opioid receptor binding and hypothalamic ß-endorphin concentration throughout pregnancy (see review: Limonta *et al.*, 1989) which are related to decreased serum LH concentrations (Dondi *et al.*, 1991). Kappa-opioid receptors have also been implicated in mediating the inhibition of LH secretion in mid-gestation in the rat (Zhen and Gallo, 1992).

There is no question that the EOP inhibit GnRH secretion in the female pig. However, until recently, the concept that noradrenergic neurons contribute to GnRH pulse generation in the pig has been in question. Early work by Parvizi and Ellendorff (1978 and 1982) demonstrated that NE administered into the central ventricular system and into discrete hypothalamic nuclei could elicit either stimulatory or inhibitory LH responses. These effects were dose dependent and, more importantly, location specific. The use of diethyldithiocarbamate (DDC) (Chang *et al.*, 1990) and N-methyl-$\dot{N}^{-1}$[1-methyl-2-propenyl] 1,2 hydrazine-dicarbothioamide (AIMAX) (Kesner *et al.*, 1987; Chang *et al.*, 1993), noradrenergic synthesis inhibitors, in the OVX gilt demonstrated that a noradrenergic input appears to be important to basal and surge secretion of LH in these animals. Most recently, Willis (1997) found that an α_1-adrenergic receptor agonist, phenylephrine, stimulated GnRH secretion from hypothalamic tissue obtained from follicular phase gilts.

How, exactly, the central noradrenergic and opioidergic neuronal systems interact to regulate GnRH secretion remains a question worth further investigation. Certainly in this study the data does not support the concept that the EOP function pre-synaptically, to inhibit noradrenergic stimulatory inputs. Phenylephrine, at a dose previously found to stimulate GnRH secretion *in vitro* (Willis, 1997), did not overcome opioidergic inhibition present in early gestation, as GnRH secretion did not increase in response to phenylephrine but did in response to naloxone. This would suggest that the EOP function post-synaptically, to directly inhibit the GnRH neurons.

Several lines of evidence from the rat suggest that the EOP inhibit noradrenergic inputs to the GnRH neurons (see reviews: Grossmann and Dyer, 1989; Kalra *et al.*, 1989). Akabori and Barraclough (1986) showed that morphine decreased LH secretion and NE concentration and turnover rates in the medial preoptic nucleus. Alternatively, naloxone

increased GnRH and NE release from perfused rat hypothalami (Leadem *et al.*, 1985). The α_1-adrenergic antagonist, phenoxybenzamine, increased the interval between hypothalamic multiunit activity (MUA), recordings known to result in a bolus of endogenous GnRH being released into the portal vessels causing the release of a pulse of LH from the pituitary. Naloxone reduced the interval between MUA volleys; however, if naloxone was administered after phenoxybenzamine it was unable to induce volleys (Nishihara *et al.*, 1991). Electrically stimulated release of ^{3}H-NE from perfused slices of rat MPOA was inhibited when morphine, ß-endorphin and met-enkephalin were added to the media. Naloxone, added to the media concomitantly, reversed the effects of morphine and ß-endorphin, but was without effect on met-enkephalin and did not affect release of ^{3}H-NE on its own (Diez-Guerra *et al.*, 1987; Dyer *et al.*, 1988). Alternatively, Heijna *et al.* (1991) found that specific μ-, κ- and δ-opioid agonists did not affected the release of ^{3}H-NE from superfused slices of rat mediobasal hypothalamus. Neurons in the MPOA that were excited by ventral noradrenergic tract stimulation were inhibited when morphine was iontophoretically applied. Morphine and naloxone in combination prevented this inhibition (Dyer and Grossmann, 1988). Both electrolytic and 6-hydroxydopamine (6-OHDA) induced lesions of the ventral noradrenergic tract resulted in decreased noradrenergic content and opioid binding in the preoptic-anterior hypothalamus of OVX-estrogen replaced rats (Dyer *et al.*, 1991). Together, these results would indicate that in the rat, an opioidergic regulation of the major noradrenergic input to the hypothalamus exists.

In the pig, there is little evidence to suggest that the EOP regulate GnRH secretion at the level of the noradrenergic neurons. Anatomical evidence certainly provides a basis for interactions between the three neuronal systems. Kineman *et al.* (1988 and 1989) identified GnRH perikarya and processes in the medial preoptic region of the porcine hypothalamus and POMC perikarya in the arcuate nucleus, with axonal projections to the MPOA. Leshin *et al.* (1996) further identified what are thought to be NE neurons in the medial region of the preoptic area (MPOA). These were identified by immunocytochemical techniques which identify the presence of the enzyme dopamine-ß-hydroxylase, which converts dopamine to NE. In contrast to the findings of the present study, Chang *et al.* (1993) suggest a presynaptic inhibition by the EOPs based on the inability of naloxone to stimulate LH secretion in AIMAX treated gilts. However, it is our feeling that if a noradrenergic synthesis inhibitor, such as AIMAX, is used and the opioidergic inhibition is then removed by naloxone, failure to demonstrate an increase in LH concentrations would be more likely due to an insufficient stimulatory NE input than to a presynaptic block on noradrenergic terminals.

A growing body of evidence provides an alternative mechanism for noradrenergic/opioidergic interactions regulating GnRH secretion. In male rats, which previously had both NE tracts lesioned, naloxone was still able to increase plasma LH concentrations. To ascertain if there were still functional NE fibers that may be mediating this effect, phenoxybenzamine was administered prior to naloxone; again, naloxone increased plasma LH in the lesioned animals but did not in the sham-lesioned animals, indicating that a functional NE system was not required for the EOP to inhibit GnRH secretion (Miller *et al.*, 1985). In several pharmacological studies it has been shown that it is the μ-opioid receptor type that mediates the inhibition of GnRH from the hypothalamus (Leadem and Kalra, 1985; Leadem and Yagenova, 1987; Walsh and Clarke, 1996). Furthermore, in rats, μ-opioid

receptors have been found in the MPOA where they occur mainly post-synaptically (Arvidsson *et al.*, 1995; Ding *et al.*, 1996), and GnRH neurons in guinea pigs possess functional μ-receptors (Lagrange *et al.*, 1995). Few EOP synapses with catecholaminergic neurons in the MPOA have been identified, although opioidergic neurons have been observed to make direct contact with GnRH neurons (reviewed by Hoffman *et al.*, 1989). GnRH is directly inhibited from GT1-1 neuronal cells via δ-opioid receptors (Maggi *et al.*, 1995). Although the GT1-1 cells represent a transformed cell line and often present receptor types not found on or involved with GnRH secretion *in vivo*, they still provide an interesting model with which to demonstrate direct neurotransmitter interactions with GnRH neurons.

In a similar experiment to the present one, Clough *et al.* (1990) found that naloxone and phenylephrine administered together had a potentiated action on GnRH release from rat POA-MBH *in vitro*, compared with either of the drugs administered separately. Furthermore, these authors reported that phenylephrine and ß-endorphin administered concomitantly had a triphasic effect on GnRH secretion, initially stimulating, then inhibiting and then stimulating GnRH again. These observations led the authors to conclude that the independent effects of both the opioid and noradrenergic compounds was highly suggestive of independent regulation of GnRH neurons by the EOP and NE systems. Immunocytochemically identified GnRH neurons, from OVX guinea pigs, were hyperpolarized by application of DAMGO, a μ-opioid agonist (Lagrange *et al.*, 1995). Electrophysiological recordings showed a rapid hyperpolarization, via the opening of inwardly rectifying potassium channels, which was reversible when naloxone was applied simultaneously. μ-receptor activation in this manner is speculated to decrease post-synaptic potentials, reducing the excitability of the post-synaptic neuron to stimulatory inputs. This provides yet another viable explanation, of how two different mechanisms of opioidergic inhibition could regulate NE neurotransmission and affect GnRH secretion. Firstly, by directly decreasing NE release through activation of pre-synaptic μ-opioid receptors and secondly, by decreasing the receptivity of GnRH neurons to stimulatory inputs via activation of post-synaptic μ-opioid receptors.

Independent regulation of GnRH secretion may also exist in the pig, in that different physiological situations, with different steroidal environments, result in differential regulation of the GnRH system by opioids. In OVX gilts, the EOP may inhibit presynaptically, via the NE neurons (Chang *et al.*, 1993) and, as was demonstrated in this study, during pregnancy, the EOP may inhibit GnRH secretion directly at the level of the GnRH neuron.

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FIGURE 6.1 Effects of the opioid antagonist, naloxone (NAL), on GnRH secretion *in vitro* from pig hypothalamic tissue. Mean (+ or - SEM) GnRH concentrations in 15 minute fractions collected during the *in vitro* perfusion of hypothalamic tissue from 6 pregnant sows, expressed as a percentage change from baseline (fractions 20-22). GnRH secretory responses to treatment with perfusion media only, NAL 2.71 X 10^{-5}M, * denotes means significantly differ from fraction 20-22 ($P \leq 0.05$), NAL 2.71 X 10^{-7}M, and NAL 2.71 X 10^{-9}M. See figure for the number of chambers represented in each treatment group.

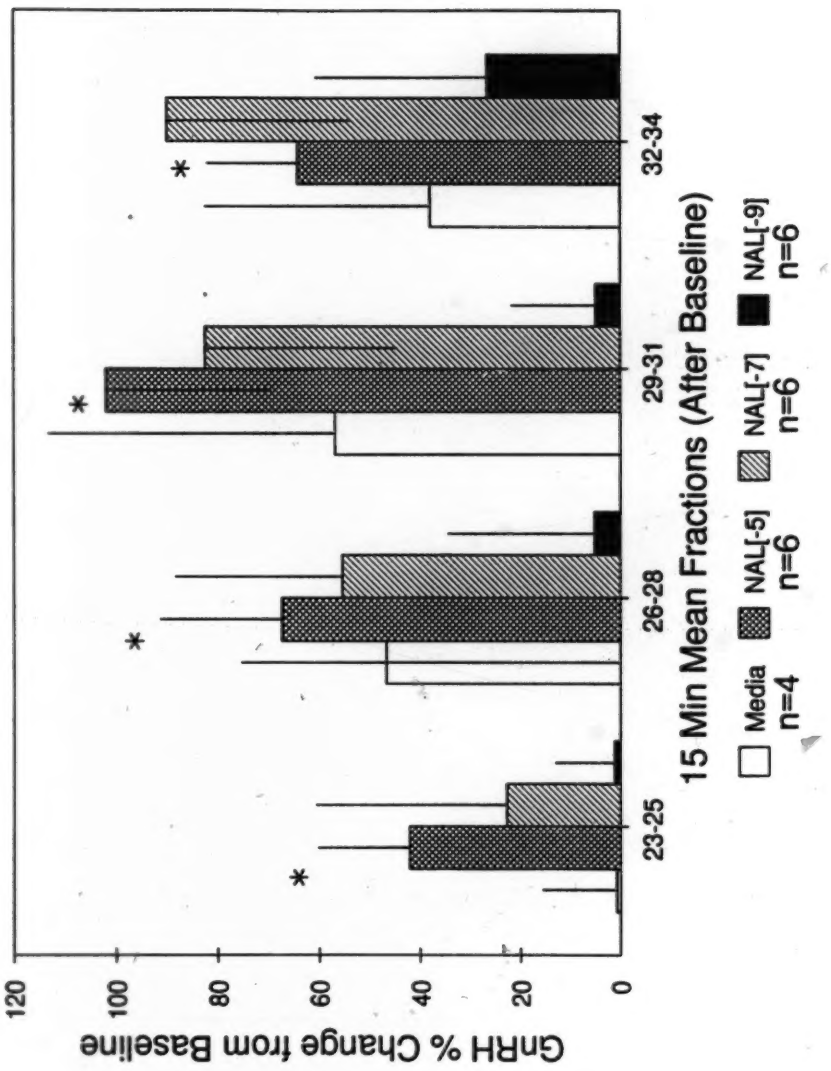

GnRH % Change from Baseline
120
100
80
60
40
20
0
*
*
*
*
23-25
26-28
29-31
32-34
15 Min Mean Fractions (After Baseline)
Media n=4
NAL[-5] n=6
NAL[-7] n=6
NAL[-9] n=6

FIGURE 6.2 GnRH profiles from 2 different perfusions. Each profile represents 2 different sows and their responses to NAL 2.71 X 10^{-5}M. In both a) and b) the baseline secretion of the 2 sows differ. However, in the response period (fractions 23-34) there is a relative increase in response to treatment in all 4 sows.

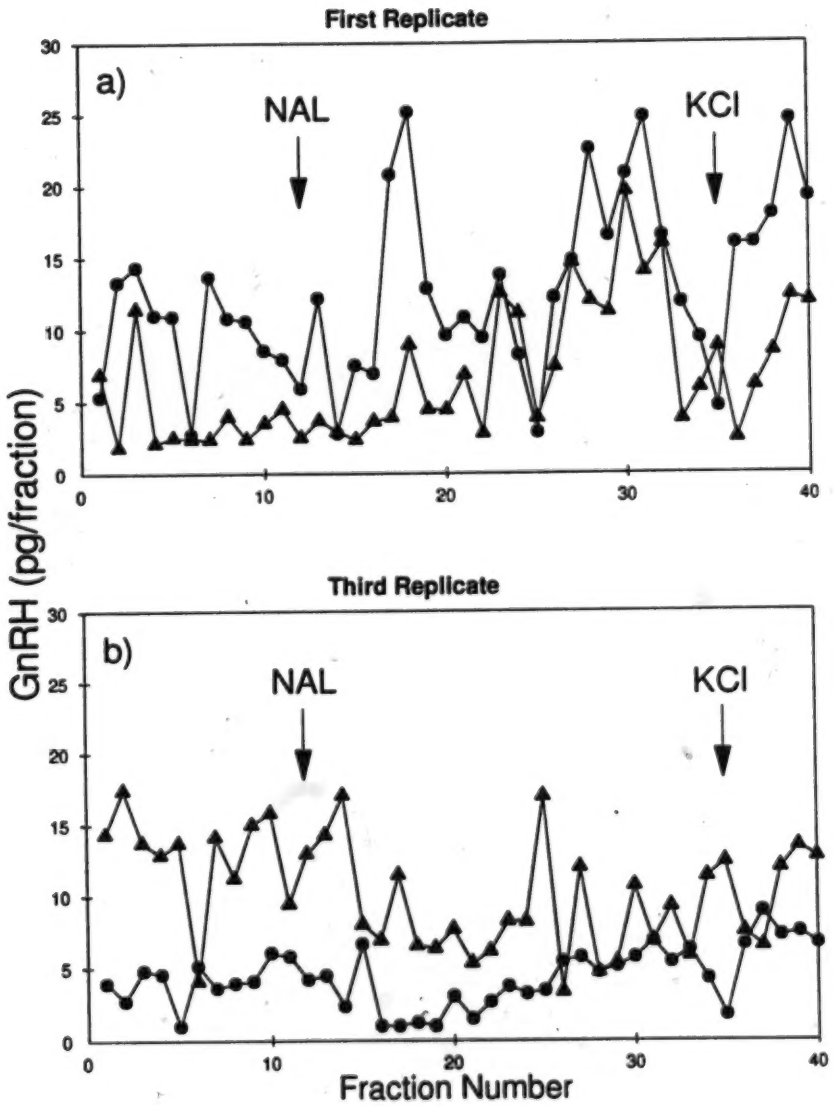

First Replicate
a)
NAL
KCl
Third Replicate
b)
NAL
KCl
GnRH (pg/fraction)
Fraction Number
0
5
10
15
20
25
30
40

FIGURE 6.3 Effects of the α_1-noradrenergic receptor agonist, phenylephrine (PHEN), on GnRH secretion *in vitro* from pig hypothalamic tissue. Mean (+ or - SEM) GnRH concentrations in 15 minute fractions collected during the *in vitro* perfusion of hypothalamic tissue from 6 pregnant sows, expressed as a percentage change from baseline (fractions 20-22). GnRH secretory responses to treatment with ascorbic acid media, PHEN 4.91×10^{-5}M, PHEN 4.91×10^{-7}M, and PHEN 4.91×10^{-9}M. See figure for the number of chambers represented in each treatment group.

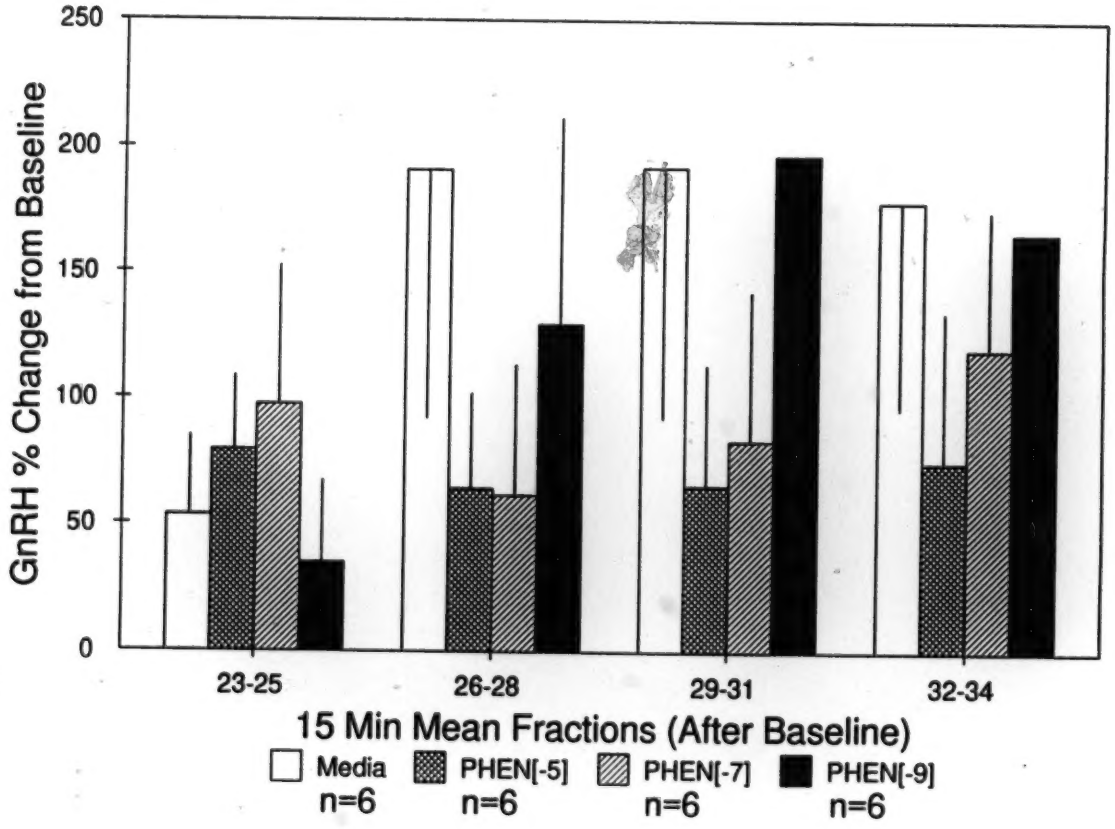
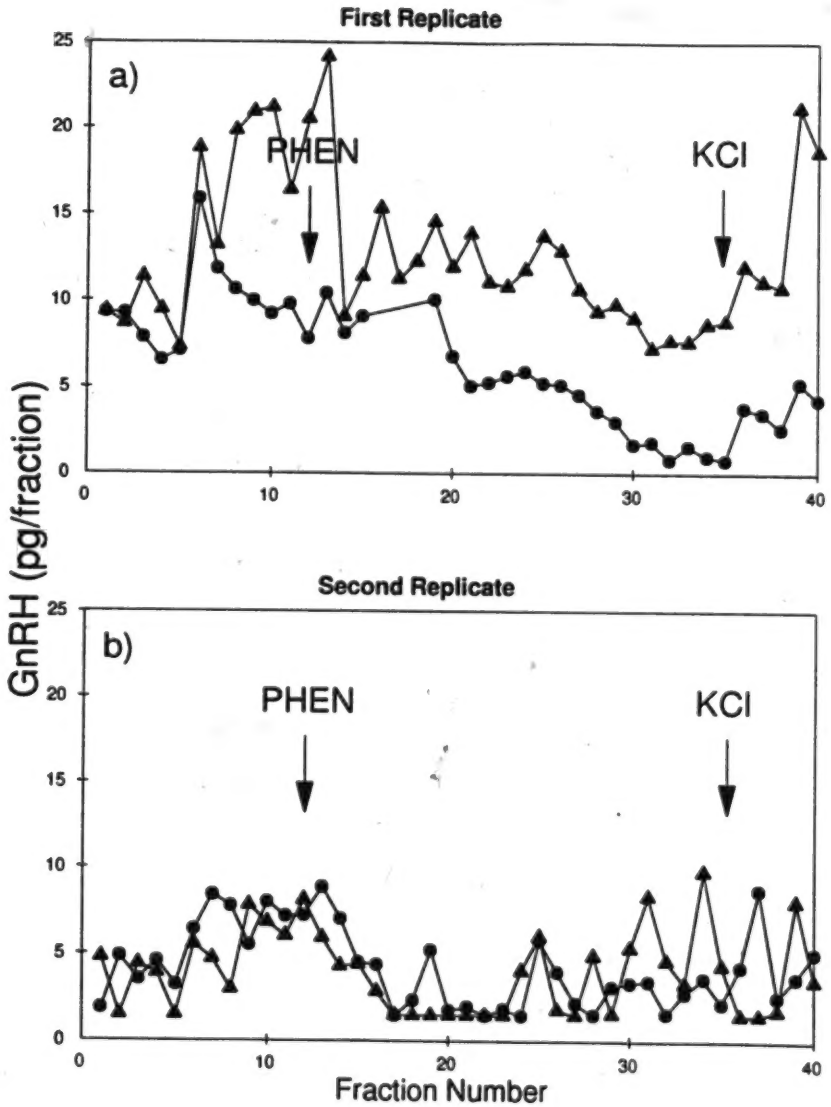

250
200
150
100
50
0
GnRH % Change from Baseline
23-25
26-28
29-31
32-34
15 Min Mean Fractions (After Baseline)
Media
n=6
PHEN[-5]
n=6
PHEN[-7]
n=6
PHEN[-9]
n=6

FIGURE 6.4 GnRH profiles from 2 different perfusions. Each profile represents 2 different sows and their responses to PHEN 4.91 X 10^{-5}M. In both a) and b) the baseline secretion of the 2 sows is similar. However, in the response period (fractions 23-34) in a) one sow shows a slight increase and then decrease, and the other sow shows a decrease in response to PHEN. In b) both sows show marginal increases in GnRH secretion in response to PHEN.


First Replicate
a)
PHEN
KCl
Second Replicate
b)
PHEN
KCl
GnRH (pg/fraction)
Fraction Number
0
5
10
15
20
25
0
10
20
30
40

CHAPTER 7
GENERAL DISCUSSION

The experiments described in this thesis were designed to further elucidate the hypothalamic inputs that regulate GnRH secretion in the female pig. Our laboratory has previously demonstrated that suckling induced inhibition of LH secretion is mediated by the endogenous opioid peptides (EOP) during established lactation in the sow (De Rensis *et al.*, 1993a). However, in the immediate post-partum period, EOP antagonism did not prevent the initial suckling-induced suppression of LH (De Rensis *et al.*, 1993a), and exogenous opioids also could not inhibit LH secretion at this time (De Rensis, 1993). With this in mind, an *in vivo* experimental approach was taken to determine if the EOP regulate LH secretion in late gestation, a period immediately prior to the period after farrowing when an opioidergic mechanism does not appear to be involved as the principal regulator of GnRH and LH secretion. Naloxone administration in late gestation did antagonize the EOP and significantly increased mean LH secretion. Studies in other species, as well as in the pig, have shown that opioidergic tone increases throughout gestation and affects LH secretion in the progesterone dominated environment (Aurich *et al.*, 1990; Dondi *et al.*, 1991; Aurich *et al.*, 1993). Szafranska *et al.* (1994) showed that the EOP regulate LH secretion in mid gestation, at day 40, but perhaps surprisingly, did not find any effect of naloxone later in gestation, at day 70. In contrast, the results of the study reported in Chapter 3 indicate that the EOP functionally inhibit GnRH/LH secretion as late as day 108 of gestation in the sow. As this immediately precedes a period where the opioids are not functional, this suggests that during parturition some change in the opioidergic regulation of LH occurs. As opioid concentrations peak during parturition (Aurich *et al.*, 1990; Dondi *et al.*, 1991), downregulation of opioidergic receptors may occur, and as a consequence the opioidergic system can not be antagonized or stimulated, until upregulation of receptors occurs some 78 hours following parturition. As suckling mediated inhibition of LH still occurs in the immediate post-partum period (De Rensis *et al.*, 1993a and 1993b) when EOP actions cannot be demonstrated, some other factor must be mediating the initial suckling-induced inhibition of GnRH/LH secretion.

Although opioidergic regulation of GnRH/LH secretion has been well documented in several physiological paradigms in the female pig (Barb *et al.*, 1986a and 1986b; Mattioli *et al.*, 1986; Armstrong *et al.*, 1988; De Rensis, 1993a; Szafranska *et al.*, 1994; Willis *et al.*, 1996), the level at which this inhibition occurs is still unknown. In other species (Matteri and Moberg, 1985; Chao *et al.*, 1986; Dragatsis *et al.*, 1995), as well as the pig (Barb *et al.* 1990), *in vitro* studies have shown a role for opioidergic regulation of LH secretion directly at the level of the pituitary. Barb *et al.* (1994) have also demonstrated an *in vitro* effect of naloxone and morphine administration on GnRH secretion from medial preoptic area tissue obtained from gilts. How the EOP function within the hypothalamus to inhibit GnRH secretion was still undetermined, but Chang *et al.* (1993) implicated an opioidergic inhibition of a stimulatory noradrenergic system. One interpretation of the results from the *in vivo* experiment described in Chapter 4 is that opioidergic regulation of GnRH secretion during lactation, and during administration of exogenous opioids after weaning, are mediated via a direct inhibition of the GnRH neuron. Alternatively, it is possible that the peripherally

administered noradrenergic agonist, phenylephrine, was unable to affect a centrally mediated system.

Originally a third *in vivo* experiment was designed to examine the factors which may be involved in the initial suckling mediated inhibition of LH secretion. However, due to the difficulties encountered in the second experiment (Chapter 4) with the peripheral administration of drugs and our inability to effectively measure centrally mediated effects on GnRH/LH secretion, an alternative approach was required. Therefore, an *in vitro* perfusion system and GnRH assay for quantifying GnRH from porcine hypothalamic tissues was developed in a series of preliminary studies, described in Appendix A.

In this series of experiments it was found that the area of the hypothalamus from which the tissue is obtained is of critical importance, as some areas have a greater secretory potential than others. These results corroborate the anatomical evidence of Kineman *et al.* (1988 and 1989) which show that the main GnRH axon tracts in the hypothalamus of the pig are located immediately adjacent to the walls of the third ventricle, and in the most lateral areas of the hypothalamus. Furthermore, equivalent secretion of GnRH from tissue of the same origin but contained in separate perfusion chambers was observed. However, tissue comprising half of a hypothalamus in one chamber was shown to secrete the same quantity of GnRH as tissue comprising one quarter of the opposite half of the hypothalamus from the same animal. Although this demonstrated that tissue representing the entire half was likely not being perfused adequately compared with tissue from one quarter of the hypothalamus, the striking similarity in pattern of secretion was of particular interest. This again raises possibilities that neural inputs to the GnRH neurons are not the pulse generator per se, but that the pulse generator resides within a population of GnRH neurons.

Studies which have chronically disrupted the noradrenergic tonus within the hypothalamus, using noradrenergic synthesis inhibitors (Herdon *et al.*, 1984), noradrenergic neurotoxic compounds (Akema *et al.*, 1990; Leonhardt *et al.*, 1991) or surgical deafferentation of the noradrenergic tracts to the hypothalamus (Clifton and Steiner, 1985), all report that these methods, while acutely interrupting LH pulsatility, do not chronically block LH pulsatility. Furthermore, in these studies, attempts to antagonize the noradrenergic system following a resumption of LH pulsatility, fails to have any effect, indicating that another neural system has assumed the role of pulse generator. A strong possibility exists that the GnRH system has assumed, or has always maintained this role. Monoclonal GnRH cell lines have demonstrated that pulsatility is an inherent characteristic of these cells (Krsmanovic *et al.*, 1992; Martinez De La Escalera *et al.*, 1992; Wetzel *et al.*, 1992). In normal GnRH neurons, synaptic contacts and non-synaptic specializations have been identified between GnRH neurons *in vivo* (Leranth *et al.*, 1985; Kineman *et al.*, 1988; Witkin *et al.*, 1995). At the very least, crosstalk between GnRH neurons is required for the synchronous firing to produce a bolus of GnRH to the portal vessels. Although Knobil's laboratory has developed the concept of the GnRH pulse generator over the years and published a vast body of knowledge on this subject, perhaps the deafferentation and electrophysiological monitoring studies which suggest that the pulse generator resides in the mediobasal hypothalamus, were more accurately assessing the GnRH neuron's ability to generate pulsatile GnRH release within their own system.

The *in vitro* system developed and described in Appendix A was subsequently used

in the experiment described in Chapter 5 to further investigate the role of hypothalamic noradrenergic systems in the generation or modulation of GnRH secretion in the porcine hypothalamus. In this study we demonstrated that an α_1-noradrenergic receptor mediated mechanism was involved in the stimulation of GnRH secretion from the medial preoptic area/mediobasal hypothalamus. These results are in agreement with findings from studies in other species (Leung *et al.*, 1982; Kaufman *et al.*, 1985; Nowak and Swerdloff, 1985; Condon *et al.*, 1989; Clough *et al.*, 1990), and with the studies in the pig which implicate the noradrenergic system in the regulation of GnRH secretion (Parvizi and Ellendorff, 1979 and 1982; Chang *et al.*, 1993). Unlike other studies which have demonstrated an inhibitory role for (Caceres and Taleisnik 1980 and 1982; Dotti and Taleisnik, 1984), or no effect of (Leung *et al.*, 1982), ß-noradrenergic mechanisms in the regulation of GnRH secretion, the present study found that the ß-noradrenergic agonist, isoproterenol, stimulated GnRH secretion at the lowest dose used. This effect was not reversible using the appropriate antagonist, propranolol, and so the observed stimulation may be artifactual. It would be of some importance to reassess this part of the experiment, using a different approach to the model, initially antagonizing GnRH secretion from the hypothalamus, and then attempting to stimulate GnRH secretion using the agonist.

As previously mentioned, the problems associated with peripherally administered drugs crossing the blood brain barrier to elicit central responses, convinced us that a more appropriate *in vitro* model was required for the further investigation of centrally mediated mechanisms of GnRH secretion. Using the *in vitro* system, the final experiment of this thesis readdressed the question asked in Chapter 4; do the EOP inhibit GnRH secretion via a stimulatory pre-synaptic noradrenergic system, or directly at the GnRH neuron itself? As opioidergic inhibition of GnRH secretion during gestation was demonstrated in Chapter 3, the availability of sows in early gestation provided an opportunity to investigate these interactions further. It was found that the EOP do inhibit GnRH secretion *in vitro* from hypothalamic tissue obtained from sows during early gestation. Furthermore, that the exogenously administered α_1-agonist, phenylephrine, could not overcome opioidergic inhibition present at this time, when the same dose of the same agonist was shown to stimulate GnRH secretion *in vitro* in Chapter 5. This indicates that the opioidergic inhibition of GnRH secretion occurs directly on the GnRH neuron. This result is in direct contrast to Chang *et al.* (1993) who showed that following *in vivo* noradrenergic depletion, naloxone was ineffective at stimulating LH release in ovariectomized gilts, which suggests that the EOP inhibit the noradrenergic stimulus at a pre-synaptic level. The difference in these two experiments, an *in vitro* versus an *in vivo* approach, and the different physiological states of the animals may have resulted in the discrepant results. Lagrange *et al.* (1995) have demonstrated that GnRH neurons possess functional opioid receptors and that application of ß-endorphin rapidly hyperpolarizes these neurons. Pre-synaptic μ-opioid receptors have also been identified by Ding *et al.* (1996). It has therefore been suggested that two mechanisms of opioidergic inhibition may occur; 1) that when post-synaptic receptors are activated, the receptivity of the neuron to incoming stimuli is reduced, and 2) when pre-synaptic receptors are activated, inhibition of neurotransmitter release occurs. These results, as well as the current study and the study by Chang *et al.* (1993) indicate that differential regulation of GnRH secretion by the opioids may occur during different physiological states.

These studies demonstrate that the *in vitro* techniques developed in this thesis provide a viable alternative to the traditional *in vivo* approaches used previously to investigate the central mechanisms regulating GnRH secretion. The benefits to using such a system have already been discussed earlier in this chapter, however, certain problems with the current *in vitro* system still exist and would need to be addressed prior to further use. The method of drug administration is of key importance, and needs to be reexamined. I would suggest that the drugs be made in a high molar concentration and administered as a pulse, which would dilute to an appropriate concentration when entering the perfusion chamber. This would decrease the lag time associated with the drug concentrations reaching a critical threshold before eliciting a response from the tissue. Reducing the perfusion chamber volume, while still maintaining adequate perfusion, would also assist in reducing the systems lag time. Furthermore, it would be important to increase the tissue stabilization period by approximately half an hour, to avoid overlapping stabilization and treatment. By modifying the *in vitro* perfusion system in such a manner, a much more efficient, effective system, would be of greater future use.

With the modifications to the system made, there are several experiments which would be considered important next steps in identifying the neural inputs which regulate GnRH secretion in the pig. Initially, the study described in Chapter 5 should be rerun, but the drugs should be administered in a pulsatile fashion. Also, during the combined agonist and antagonist trials, administering the antagonist first, followed by the agonist to attempt to stimulate GnRH secretion, may be a more effective method to demonstrate tissue responses to treatments. Furthermore, it would be interesting to reexamine noradrenergic and opioidergic interactions in the luteal phase gilt to see whether the results differ from those obtained from gestating sows, in Chapter 6, or whether they are more comparable to those of Chang *et al.* (1993). Alternatively, the *in vitro* noradrenergic/opioidergic interactions could be reexamined in ovariectomized, steroid-primed and unprimed gilts to determine the importance of the ovarian steroid background to centrally mediated regulation of GnRH secretion. While these studies would provide valuable information regarding the noradrenergic and opioidergic inputs to the GnRH pulse generator in the pig, future studies to examine other neural inputs, such as GABAergic or excitatory amino acid systems, provide many more exciting possibilities.

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APPENDIX 1
The Development of an *In Vitro* Perfusion System and Assay for Assessing GnRH Release from Porcine Hypothalami

INTRODUCTION

In studies with several different species, *in vitro* perfusion of hypothalamic tissue has been used successfully to elucidate the role of peptidergic and classical neurotransmitters within the hypothalamus. In particular, perfusion has helped identify neuronal inputs which regulate the GnRH pulse generator, located in the hypothalamus (Clough *et al.*, 1988, norepinephrine; Herbison *et al.*, 1989, gamma-aminobutyric acid; Meyer, 1989, serotonin; Barb *et al.*, 1994, opioids; Navarro *et al.*, 1994, excitatory amino acids; Urban *et al.*, 1996, neuropeptide Y). Many of these studies have been pharmacological in nature. The use of an *in vitro* model has allowed a greater in depth look at hypothalamic regulation of GnRH secretion by overcoming the problems associated with delivering drugs peripherally and trying to determine if there was a centrally mediated response. In fact, many of the receptor agonists and antagonists available to researchers do not even cross the blood brain barrier.

In pigs, until recently, the use of alternative research techniques to overcome central administration of drugs or to directly quantify GnRH secretion from the hypothalamus, has been limited. Only a few studies exist in which techniques commonly used in small animal experiments have been utilized in the pig. Parvizi and Ellendorff (1978 and 1982) administered noradrenergic drugs directly into the hypothalamus via the ventricular system and also directly applied norepinephrine (NE) to specific regions within the hypothalamus of both male and female Göttingen miniature pigs. These studies demonstrated that there is both a stimulatory and inhibitory component to noradrenergic regulation of LH secretion. Estienne *et al.* (1990) have also used intraventricular administration of morphine to show an opioidergic regulation of LH in ovariectomized (OVX) gilts. Leshin *et al.* (1992) modified that technique to allow push pull perfusion of the anterior pituitary gland to demonstrate an association between GnRH and LH pulsatility.

Other classic *in vitro* techniques have also recently been utilized to further identify mechanisms underlying the relationships between GnRH and LH secretion. Isolated pig pituitary cell cultures have provided evidence for catecholaminergic (Pi-Hsueh, 1989), opioidergic (Barb *et al.*, 1990) and excitatory amino acid (Barb *et al.*, 1993) modulation of pituitary gonadotropin release. Static incubations of medial preoptic area (MPOA) and mediobasal hypothalamic (MBH) explants yielded discouraging results, as GnRH could not be measured in the media (Sesti and Britt, 1993). However, this could have been due to poor diffusion of GnRH out of the tissues or to very low GnRH secretion rates in hypothalamic tissue obtained from early weaned and lactating sows. Conversely, GnRH was readily measurable in media collected from a static incubation of stalk median eminence tissue which had been stimulated with KCl (Sesti and Britt, 1994), suggesting that the area of the hypothalamus from which the explants are isolated plays an important role in the success of the methodology. Finally, Barb *et al.* (1994) successfully perfused hypothalamic-preoptic

area (HPOA) explants from cyclic and OVX gilts to show that GnRH secretion is stimulated by naloxone and inhibited by morphine administration *in vitro*.

Although our lab has previously been very successful using *in vivo* models to investigate the mechanisms regulating GnRH/LH secretion in the female pig (Foxcroft *et al.*, 1987; Cosgrove *et al.*, 1991 and 1993; De Rensis *et al.*, 1991, 1993a and 1993b; Willis *et al.*, 1996; Zak *et al.*, 1997), we have never before attempted to use an *in vitro* model to investigate central mechanisms regulating GnRH/LH secretion. This approach would allow neural inputs to the GnRH system to be determined with the use of appropriate drugs, thus aiding our understanding of the central regulation of GnRH secretion and the application of this information to clinical manipulations of fertility in the pig. The successful set-up of a perfusion system would allow us direct access to the GnRH pulse generator and thus enable us to overcome difficulties with peripheral drug administration.

MATERIALS AND METHODS

General Methodologies

Animals and Tissue Collection

A total of 21 Camborough PIC male castrates (barrows) of approximately 110 kg b.w. from the University of Alberta Swine Research Unit were slaughtered and brain tissue collected for *in vitro* assessment of GnRH release. Male castrates were initially used in these preliminary experiments to facilitate the development of the *in vitro* perfusion system and GnRH assay; with the negative feedback of testosterone removed, GnRH secretion is essentially "free running", thus enabling quantification of GnRH from an *in vitro* system.

Barrows were slaughtered at a commercial abattoir by electro stunning and exsanguination. Craniotomy and removal of the entire brain occurred within 10 minutes of electro stunning. A block of tissue containing the entire HPOA was then excised on a chilled surface, using a brain knife (see Figure A.1). Cuts were made 5 mm rostral to the optic chiasm and immediately rostral to the mamillary bodies. These cuts produced a slice of tissue approximately 10 mm thick, which was then placed with the posterior surface facing up. HPOA explants were further isolated from this slice of tissue using a razor blade to cut laterally at the thalamic boundary (top of the third ventricle was used as a marker) and further cut sagitally at the third ventricle, producing two halves with a "tail" of cortical tissue to aid in stabilizing the tissue while producing hypothalamic slices for perfusion. Median eminence (ME) tissue was not included. Each half was then sagitally sliced into 500 µm sections with a tissue slicer (Stoelting Co. Wood Dale, IL) and tissue from each half was placed in separate transport vials (Fig.A.2a). Hypothalamic nuclei included in these slices are represented in Figure A.3. Tissue was transported back to the lab in these individual vials containing chilled, continuously oxygenated (5% CO_2, 95% O_2) perfusion medium.

Perfusion System

Tissue slices from each half of each HPOA explant were placed into perfusion chambers with a 2.3 ml capacity (25mm SWINNEX filters, Millipore Corporation, Bedford MA) and maintained in a water bath at 37° C. Perfusion medium consisted of Delbecco's

Modified Eagle's Medium and Ham's Nutrient Mixture F-12 (DME/F12; pH 7.3; 1.2 g $NaHCO_3$) with addition of 0.1% BSA, 0.1mM bacitracin and 5 mg kanamycin sulfate/l (Sigma Chemical Co., St. Louis, MO) as described by Barb *et al.* (1994), but excluding 5 mg amphotericin B from their original formulation. Medium was oxygenated (95% O_2-5% CO_2), warmed to 37° C and pumped through tissue chambers at the rate of 100 µl/min. Fractions were collected at 5 min intervals (500 µl) into polypropylene vials, snap frozen and stored at -70° C until assayed for GnRH. 1 min depolarizing pulses of 1.38M KCl were introduced from a separate media reservoir, to produce final chamber concentrations of 60 mM KCl, to assess tissue responsiveness. The pattern in which these pulses were introduced to the chambers varied over the course of protocol development (see individual sections for details). However, in all replicates, chambers received a 1 min pulse of KCl at the beginning of fraction 35 as a means of assessing tissue viability at the end of the perfusion.

GnRH Assay Development

GnRH acetate salt (Sigma) was iodinated using the method of Nett and Adams (1977). GnRH was quantified in media samples using the single antibody radio immunoassay described by Sesti and Britt (1993) and antibody kindly supplied by Dr. J.H. Britt (North Carolina State University, Raleigh, NC). As the perfusion protocol was developed, minor modifications were made to the original assay procedure. The main problem with our use of this assay was that many of the fractions collected from the barrows contained GnRH concentrations which were below the sensitivity of the assay. Initially we tried to improve sensitivity by increasing the antibody dilution and decreasing the amount of radio label added to each assay tube.

The original protocol required that the antibody be diluted to 1:80,000 and the counts per minute per tube (cpm/tube) were between 15,000-20,000. Standard curves were assayed with various dilutions of antibody; 1:80,000, 1:120,000, 1:160,000, and 1:320,000, with 15,000 cpm/tube. As none of the dilutions changed the sensitivity of the assay (Fig.A.4a), the 1:80,000 antibody dilution was retained as it gave the highest total binding. Another series of standard curves were assayed, using different amounts of label; 5,000, 10,000, 15,000 and 20,000 cpm/tube with a 1:80,000 antibody dilution. The standard curve containing 5,000 cpm/tube showed a slight shift in sensitivity (Fig.A.4b) and had the highest total binding. However, combining these results, using 5,000 cpm/tube and varying the antibody dilutions as previously described and counting each tube for 5 minutes, did not result in any further increases in assay sensitivity (Fig.A.4c). It was therefore decided that the assay should be run using an antibody dilution of 1:80,000 and 10,000 cpm/tube and counting for 5 min per tube.

The alternative to manipulating the assay and standard curve was to concentrate the samples. Media collected from the transport vials was pooled and determined to contain GnRH leaked from the hypothalamic tissue during transport from the abattoir. Pooled transport media (TM) was initially assayed at 50, 100 and 200 µl volumes to determine parallelism. It was ascertained that dilutions of the TM ran parallel to the standard curve (Fig.A.5a). 2.4 ml of TM was dried down and reconstituted with 800 µl of assay buffer. The reconstituted TM was assayed at 50, 100 and 200 µl volumes; although parallel to the standard curve, there was only a twofold, rather than threefold, increase in dose in response to having been concentrated (Fig.A.5a), this may have been due to destruction of the native

peptide during the concentrating process. See Figure A.5b for the full range of TM dilutions used to ascertain parallelism. Based on parallelism of 200 µl of TM, 200 µl of media sample was added to each unknown tube, omitting the 100 µl of assay buffer, so that total reaction volume remained the same. This system was sufficiently sensitive to measure the majority of samples collected during perfusion.

The intra- and inter-assay coefficients of variation, reported for assays from Parts 4 and 5 of these trials (when assays were standardized to 1:80,000 antibody, 10,000 cpm/tube, 200 µl of unknown and 5 min count time), were 12.92% and 11.48%, respectively. The overall sensitivity of these assays was 0.53 pg/tube.

Perfusion Development

Part 1: The first 2 perfusion replicates were used to determine if GnRH could be measured in the collected fractions. The results from these initial replicates are discussed in the assay development section of this paper. Following a 60 min tissue stabilization period, in which fractions 1-11 were collected, a series of KCl challenges were administered. 1 min depolarizing pulses of 1.38M KCl were administered at the start of fractions 12, 24 and 36 (60, 120 and 180 min after the start of perfusion, respectively). Tissue responses to depolarization decreased with each subsequent KCl pulse. It was also determined at this time that the perfusion system had an inbuilt lag time of approximately 15 minutes, the time required for KCl to leave the media reservoir and the fraction containing the response to be collected. Transport media (TM) was also assayed to determine if hypothalamic halves leaked an equivalent amount of GnRH from the sliced tissue during transport to the lab. Fresh media was assayed to determine if it cross reacted in the assay. There was no binding associated with these media tubes. Following perfusion, tissue contained in the chambers was weighed. There was a lot of variation between symmetrical hypothalamic halves most likely due to different rates of GnRH secretion *in vitro* and differences in residual media content in the tissue. Due to the difficulties associated with weighing the live tissue upon arrival at the lab it was decided to forgo tissue weights and expression of GnRH secretion on per weight basis. See Part V for results relating to secretion on a per weight basis.

Part 2: To determine if the decline in tissue response to KCl seen in the previous replicates was due to depletion of GnRH from the tissues or a decrease in tissue viability with time, 1 min pulses of KCl were administered at the start of fractions 12, 18, 24, 30 and 36 (60, 90, 120, 150 and 180 min after the start of perfusion, respectively). Again, tissue responses to depolarization decreased with each subsequent KCl pulse. Following perfusion, hypothalamic tissues were homogenized (Ultra-Turrax T25, IKA-labortectinik, Janke and Kunkel, Germany) in 2 ml of perfusion medium and centrifuged at 1,500 x g and 4°C for 30 min. The supernatant was decanted and re-centrifuged for a further 15 min. The resulting supernatant was stored at -70°C until assayed for GnRH. Hypothalamic extract (HE) was initially assayed at a volume of 100 µl but due to the high concentration of GnRH contained in the HE samples they had to be included in the assay in 50 µl volumes. The results indicated that there were still large stores of GnRH contained in hypothalamic tissue at the end of the perfusion, although it can not be ascertained whether this was in the releasable pool of GnRH, and that the decreased response to subsequent depolarizations was probably due to decreased tissue viability. Furthermore, following a paired t-test analysis, there was no difference

(P=0.68) between hypothalamic halves from each barrow as to the amount of GnRH contained in their respective HE (Fig.A.6a). Variability between halves was therefore due to the amount of GnRH actually secreted from the tissues while in perfusion. There was no difference (P=0.26) between GnRH concentrations in the TM from each barrow between halves (Fig.A.6b).

Part 3: Visual appraisal of GnRH secretory profiles from Parts 1 and 2 indicated that GnRH secretion was still quite high and variable between barrows, even after a 60 min tissue stabilization period. In this replicate, a 90 min stabilization period was attempted, followed by a 1 min KCl pulse at the start of fraction 18, in an attempt to mimic the addition of a noradrenergic drug to the system at this time point in the actual experimental protocol, to follow. The longer equilibration time allowed a steady pretreatment baseline to be attained prior to KCl treatment at the start of fraction 18.

Part 4: Due to the large quantity of tissue contained in each hypothalamic half, the relatively small size of the chambers, and problems with tissue being "sucked" out of the chambers and into the perfusion lines, a fine nylon filter was fitted over the exit from the chambers. The question of whether the tissue was being adequately perfused was also addressed. To address this problem, smaller amounts of hypothalamic tissue were included in each chamber. Two barrows were slaughtered for each of 3 replicates, and the HPOA removed as previously described. One half of each barrow's hypothalamus was sliced into 500 µm sections and all slices included in a single transport vial. For the remaining half, however, the tissue slices were alternated between 2 transport vials, in effect each vial representing a quarter of a hypothalamus. As the slices were alternated between vials, the nuclei contained in these slices should be fairly homogenous between the two vials (see Figures A.2b and A.3 for schematics). As a result of this sectioning method, two barrows created six perfusion chambers; two chambers containing a sliced half of hypothalamus from each barrow and four chambers containing a sliced quarter of hypothalamus. Following a 90 min stabilization period, a 1 min pulse of 1.38M KCl was administered at the start of fraction 18. Another 1 min pulse of KCl was administered at the start of fraction 35 to assess tissue viability at the end of the perfusion. In situations where active GnRH secretion was occurring at the time of the KCl challenge, the tissue was deemed viable and data were included in further statistical analysis. However, if GnRH secretion was not evident, or was below assay sensitivity, in the period immediately preceding the KCl challenge, then an increase of 2 standard deviations above the mean of the 3 samples immediately preceding the peak response fraction was taken as tissue viability and data were included in further statistical analysis.

To enable analysis (reduce the noise associated with the system) GnRH concentrations across the 5 min fractions 2-40 were transformed into 15 min mean fractions, creating 13 time periods. Fraction 1 was excluded due to the large variation between chambers upon start-up of the system. The sequential 15 min GnRH means for halves of hypothalami were then compared against the means for quarters of hypothalami, across time (times 1-13) (PROC GLM, SAS statistical package, 1988) using day (day the perfusion was run), barrow and section (half vs quarter of hypothalamus) as class variables and barrow X section as the error term. ANOVA for repeated measures was used to make polynomial contrasts across time to determine if there was a pattern of GnRH secretion during perfusion.

During the entire perfusion period (fractions 1-37; analysis time points 1-12) there was

no significant difference in GnRH secretion between hypothalamic halves and quarters. As the secretion rates were the same between the 2 amounts of tissue (see Figure A.7), the results would suggest that the hypothalamic halves were not being adequately perfused in this system, thus not allowing GnRH to diffuse out of the tissue. At time point 13 (fraction 38-40), which represents the response to KCl stimulation at the beginning of fraction 35, there was a difference (P=0.03) in GnRH secretion between halves and quarters; the hypothalamic halves secreting more GnRH than the quarters ((mean ± SEM) 5.41 ± 1.96 pg/15 min mean vs 3.70 ± 1.79 pg/15 min mean, respectively). This difference between sections was not evident at the earlier time point representing a response to KCl stimulation. Polynomial contrasts for repeated measures detected a significant (P=0.008) linear effect of time on GnRH secretion regardless of the amount of tissue placed in the chamber, and GnRH secretion declined from fraction 1 through 40. A paired t-test established that there was no difference (P=0.74) between hypothalamic halves from each barrow as to the amount of GnRH contained in their respective HE (Fig.A.6a) following perfusion. Again, any variability between halves would be due to the amount of GnRH actually secreted from the tissues while in perfusion, demonstrating that both halves and quarters secrete an equal amount of GnRH. There was no difference (P=0.09) between GnRH concentrations in the TM from each barrow between halves and quarters (Fig.A.6b), although this was almost significant and was likely due to a larger amount of severed neurons leaking GnRH into the TM.

Part 5: In an attempt to further reduce the amount of tissue used in perfusion, yet another method of sectioning the hypothalamic halves was attempted, based on regional differences of GnRH-containing nuclei within the hypothalamus. One barrow was slaughtered for each of 4 replicates, and the HPOA removed as previously described. One half of the hypothalamus was sliced into 500 µm sections and the first 4 slices immediately lateral to the third ventricle were placed in 1 transport vial, the next 4 slices in a different vial and the last 4 slices in another vial (total of 12 hypothalamic slices in 3 vials). For the remaining half, the tissue was also sliced into 500 µm slices but only 2 slices were placed into individual vials (total of 10 hypothalamic slices in 5 vials; remaining tissue from this half had to be discarded due to constraints by the number of chambers available for perfusion) (see Figure A.2c for schematic). As a result of this sectioning method, 1 barrow created 8 perfusion chambers; 3 chambers containing 4 slices of hypothalamus and 5 chambers containing 2 slices of hypothalamus. Following a 90 min stabilization period, a 1 min pulse of 1.38M KCl was administered at the start of fraction 18. Another 1 min pulse of KCl was administered at the start of fraction 35 to assess tissue viability at the end of the perfusion.

To facilitate analysis (reduce the noise associated with the system) GnRH concentrations across the 5 min fractions 2-40 were transformed into 15 min means, creating 13 time periods. Fraction 1 was again excluded due to large variations between chambers upon start-up of the system. Using least square means (lsmeans) analysis, 15 min GnRH means for each perfusion chamber were compared against one another, across time (times 1-13) (PROC GLM, SAS statistical package, 1988), using barrow and chamber (whether it contained 2 slices or 4 slices of tissue) as class variables, and using barrow X chamber as the error term. This analysis identified any differences in GnRH secretion between the subsequent 2 slices of tissue across one half of the hypothalamus, or differences between the subsequent 4 slices of tissue on the opposite half of the hypothalamus, at different time points during the

perfusion. This analysis did not allow comparison between chambers that contained 2 slices of tissue with chambers containing 4 slices of equivalent tissue from the opposite hypothalamic half. To address the latter analysis, chambers were designated 1a, 1b, 1c, 1d and 1e for chambers containing 2 slices of tissue (moving laterally from the 3rd ventricle 1a-1e, therefore, making the designation equal to the location of the tissue within the hypothalamus) and 2A, 2B and 2C for chambers containing 4 slices of tissue (also moving laterally from the 3rd ventricle 2A-2C). Using least square means analysis with this designation, GnRH secretion over 15 min from 1a + 1b = 2A, 1c + 1d = 2B and 1e was compared against 2C, although the amounts of tissue were not equivalent due to chamber number constraints (ie. the equivalent of tissue location 1f was discarded and so the comparison 1e + 1f = 2C, could not be made), comparisons were made at each time point (1-13), with barrow and location (origin of the slices within the hypothalamus; 1a-1e or 2A-2C) as class variables and barrow X location in the error term. ANOVA for repeated measures was used to make polynomial contrasts across time to determine if there was a pattern of GnRH secretion during perfusion.

As expected, during the stabilization period (fractions 1-17; analysis time points 1-6) GnRH secretion was highly variable between sections 1a-1e and 2A-2C, and the results do not reveal anything important, therefore there will be no discussion of statistical results. Following KCl stimulation during fraction 18, there were significant ($P \leq 0.03$) differences in GnRH secretion between hypothalamic sections 1a-1e and 2A-2C at various time points through the remainder of the perfusion (fractions 20-40) (Table A.1). At no time points following the stabilization period did rate of GnRH secretion differ between 1a + 1b = 2A, 1c + 1d = 2B and 1e = 2C, indicating that the 2 chambers containing 2 slices of equivalent tissue to the 1 chamber containing 4 tissue slices secreted the same amount of GnRH at all time points. The results from these analyses also demonstrate a regional difference within the hypothalamus as to where GnRH secretion occurs (GnRH neurons and terminals). The results are particularly clear with the chambers containing 4 slices of tissue: the greatest number (based on secretion rate) of GnRH neurons are contained immediately lateral to the third ventricle, area 2A, and there are relatively few GnRH neurons in the medial area of the hypothalamus, area 2B, and there are more GnRH neurons located in the most lateral edge of the hypothalamus, area 2C. However, based on the secretion rate of GnRH from area 2C, there are less neurons in this area than in area 2A, but more neurons than in area 2B (Fig.A.3). Polynomial contrasts for repeated measures detected a significant linear ($P=0.02$) and quadratic ($P=0.008$) effect of time on GnRH secretion regardless of the amount of tissue in the chamber or the original location of the tissue within the hypothalamus. Irrespective of these factors, GnRH secretion declines over time.

Part 6: This section includes elaboration of some of the methods and statistics found in Chapters 5 and 6 containing the cyclic gilt and pregnant sow data (see Chapters 5 and 6 for basic Materials and Methods). Based on the data in Part 5 of the preliminary studies, only the 4 slices immediately lateral to the third ventricle from both hypothalamic halves were included in the perfusion, combining slices 1 and 3, and 2 and 4 from each half (Fig.A.1f), in separate transport vials. Once in the lab, the two tissue slices from each transport vial were transferred to individual perfusion chambers, thereby creating eight chambers from the two animals slaughtered on each occasion. The tissue was allowed to stabilize for 120 min (1 hour of

washout, no samples collected and 1 hour of collection, fractions 1-11) prior to any drug treatments being administered.

Once the GnRH secretory profiles from the gilt and sow studies had been visually appraised, it was evident that the secretory pattern differed from that of the "free-running" hypothalamus of the barrow. Although the stabilization period had actually been extended by 30 min from that of the preliminary work, it was found that a stable baseline was not attained until 160 min after the start of perfusion, or by the collection of fraction 20. This presented the problem of the pre-treatment baseline occurring during drug administration (started at fraction 12). However, in both the noradrenergic and noradrenergic/opioidergic drug trials the drugs were prepared in different molar concentrations, and were administered from media reservoirs at these concentrations. As well as the time required for a substance to flow through the system from the media reservoir to the collection vial, as discussed in Part I, an inbuilt lag time would also exist due to the system flow rate being set at 100 µl and the perfusion chamber volume being 2.3 ml. These factors would account for a lag in the system before drug threshold concentrations for stimulation would be reached within the chambers. These threshold levels would be expected to occur during fraction 17-18 but due to the fluid dynamics of the system (100 µl entering and exiting the chamber simultaneously) it is reasonable to expect threshold concentrations to be attained and a measurable response to be collected after these fractions, and in fact that was the case.

As previously discussed, GnRH concentrations from 5 min fractions 2-40 were transformed into 15 min mean fractions. As a baseline of GnRH secretion had been established by fraction 20, the 15 min fraction 20-22 was used as a pretreatment baseline, with which relative changes in GnRH responses to various drug treatments could be compared. Mean GnRH responses in sequential 15 min intervals were determined for 60 min after stimulation (fractions 23-34). Data were expressed as percent change from baseline to reduce the effect of variation between chambers in absolute GnRH concentrations.

In the combined noradrenergic agonist/antagonist drug treatment trials described in Chapter 5 , the noradrenergic receptor agonists, phenylephrine (α; PHEN 4.91 X 10^{-5}M) or isoproterenol (β; ISO 4.04 X 10^{-9}M) were introduced into the system at the start of fraction 1 and continued through to fraction 34. When the GnRH profiles for each respective agonist were appraised, it was clear that by the end of 1 hour (fraction 12) GnRH secretion was sufficiently maintained to allow the appropriate antagonist (prazosin or propranolol, respectively) to reduce GnRH secretion. Data was transformed into 15 min means, however the baseline with which to compare responses to antagonist treatment was determined to be fraction 11-13. Mean GnRH responses in sequential 15 min intervals were determined for 105 min after antagonist treatment (fractions 14-34). Data were again expressed as percent change from baseline. ANOVA for repeated measures (PROC GLM, SAS statistical package, 1988) was applied to the data. When overall treatment effects were significant, linear contrasts were used to compare responses over specific 15 min time periods with the baseline.

As a point of interest, and to confirm that the appropriate baseline was used in the final analysis, fraction 20-22 was used as the baseline for the data from the group which received agonist only from fractions 1-34 (no antagonist added at fraction 12). All 15 min mean fractions prior to the baseline (fractions 2-19) and after the baseline (fractions 23-34)

were compared against the results from the analysis of the same dose group from the agonist only trials (PHEN; 4.91 X 10^{-5}M or ISO; 4.04 X 10^{-9}M). It appeared that administering PHEN an hour earlier had indeed changed the pattern of GnRH secretion seen when PHEN had been administered beginning at fraction 12, although this was not the case for ISO. For PHEN, these results indicated that GnRH secretion was maintained through a period in which it had declined in the agonist only trial, which confirmed, both our statistical results and that the correct baseline (fraction 11-13) had been used in the final analysis.

DISCUSSION

The results from the preliminary *in vitro* studies indicate that porcine GnRH can be collected from and measured in hypothalamic tissues. Porcine GnRH has previously been measured directly in homogenized hypothalamic tissues obtained from early weaned and lactating sows (Sesti and Britt, 1993) and in samples collected from perfused HPOA tissue from cyclic and OVX gilts (Barb *et al.*, 1994). Similar to the static incubations performed by Sesti and Britt (1993), many of the samples initially collected in Part 1 of these trials were below the sensitivity of the GnRH assay. However, following a switch to 200 µl of sample per assay tube, almost all samples were above the assay sensitivity. The 90 min stabilization period used in the barrow work appears adequate for the tissue to reach a stable baseline, and is of the same duration used by Barb *et al.* (1994). During work which includes tissue from follicular phase gilts, a longer stabilization period would be more appropriate.

There was no difference in the amount of GnRH secreted over the period of perfusion between hypothalamic halves and quarters. This indicates that the hypothalamic halves were not being perfused adequately, and not therefore allowing for proper oxygenation and diffusion of media into the tissues and GnRH out of the tissues. Further reduction in the amount of tissue used had no detrimental effects on the outcome of the perfusion but it was determined that there are regional differences in the amount of GnRH secreted from different hypothalamic areas. Kineman *et al.* (1988) located GnRH perikarya in the MPOA of the porcine hypothalamus. The main GnRH axonal tracts to the median eminence and portal vessels coursed along the third ventricle and through the lateral area of the hypothalamus. These areas correspond well to the higher rates of GnRH secretion seen in tissue slices 2A and 2C, from Part 5 of this study, and explains the relatively low secretion of GnRH from area 2B, the middle tissues of the hypothalamus. Expressing GnRH responses on a per weight of hypothalamic tissue basis is of no use unless the entire half of the hypothalamus is perfused and weighed as the tissue is not homogenous for GnRH secretion across the hypothalamus. The major risk associated with only using certain areas of the hypothalamus is the exclusion of nuclei that may contain neurons which would normally affect GnRH secretion. In the subsequent gilt perfusions, which only included 2 slices of tissue immediately lateral to the third ventricle, GnRH perikarya in the MPOA and the axon tracts along the third ventricle would have been included in the perfusion. As well, the noradrenergic inputs impinging on the GnRH neurons would also have been included. Leshin *et al.* (1996) identified dopamine-β-hydroxylase containing neurons, putative noradrenergic synthesizing neurons, in the MPOA. Given that much less tissue can be used for perfusion in this system it would be

worthwhile to consider reducing the volume of the chambers to reduce the inbuilt lag times associated with the low flow rate and the large chamber volume.

In Part 6 discussed in this Appendix, noradrenergic drug administration was accomplished by physically switching intake lines to each chamber to media reservoirs containing the drugs at actual molar concentrations. This presented problems associated with the tissues attaining baseline secretion during the drug administration period, but prior to the chamber concentration of drugs reaching a threshold concentration for stimulation of a GnRH secretory response. In future studies of this nature, a different method of drug administration may be more favourable, combined with a longer stabilization period. Likely the most efficient method of administering drugs would be in a pulse, similar to the 1 min pulse of 1.38M KCl, which resulted in a final chamber concentration of 60 mM. Noradrenergic or opioidergic drugs could be made in a high molar concentration and administered in a pulse which would dilute to a final chamber concentration of 10^{-5}M, for example. Alternatively, a combination of pulse administration and a reservoir change to actual concentration could be used for sustained administration of drug. Furthermore, in the combined agonist and antagonist trials described in further detail in Part 6, in future studies it may be more effective to administer the antagonist first, during the period of active GnRH secretion during the equilibration period, and then attempt to stimulate GnRH secretion with the agonist.

These preliminary studies demonstrated that an *in vitro* perfusion system and GnRH assay provide an alternative model which can be used successfully to further investigate the central regulation of GnRH in the porcine hypothalamus. This allowed us direct access to the GnRH pulse generator, thus overcoming difficulties associated with using *in vivo* models in this area of research. Future trials with this system could potentially investigate further neural inputs to the porcine GnRH pulse generator, and the effect that differing steroidal milieus may have on them. Following the recommendations in this paper, the perfusion system would become more effective for use in future trials.

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FIGURE A.1 A: ventral surface of the gilt's brain; the 2 lines indicate the cuts made with the brain knife to isolate the block of tissue, approximately 10 mm in thickness, extending from a line 5mm rostral to the optic chiasm (oc) to a line immediately rostral to the mamillary bodies (mb), that contains the entire hypothalamic-preoptic area (HPOA). B: the slice of tissue isolated in A, facing posterior side up. C: the dotted line indicates the lateral cut with a razor blade at the thalamic boundary (th), using the top of the third ventricle (v) as a marker, to further isolate HPOA explants. D: shows the isolated explants with "tails" (t) of cortical tissue. HPOA halves (E) were separated with a razor blade by cutting sagitally at the third ventricle. F: representation of the 500 µm thick tissue slices included in the perfusion in Part VI (see text for further explanation); numbers represent the number of chambers created from this slicing technique.

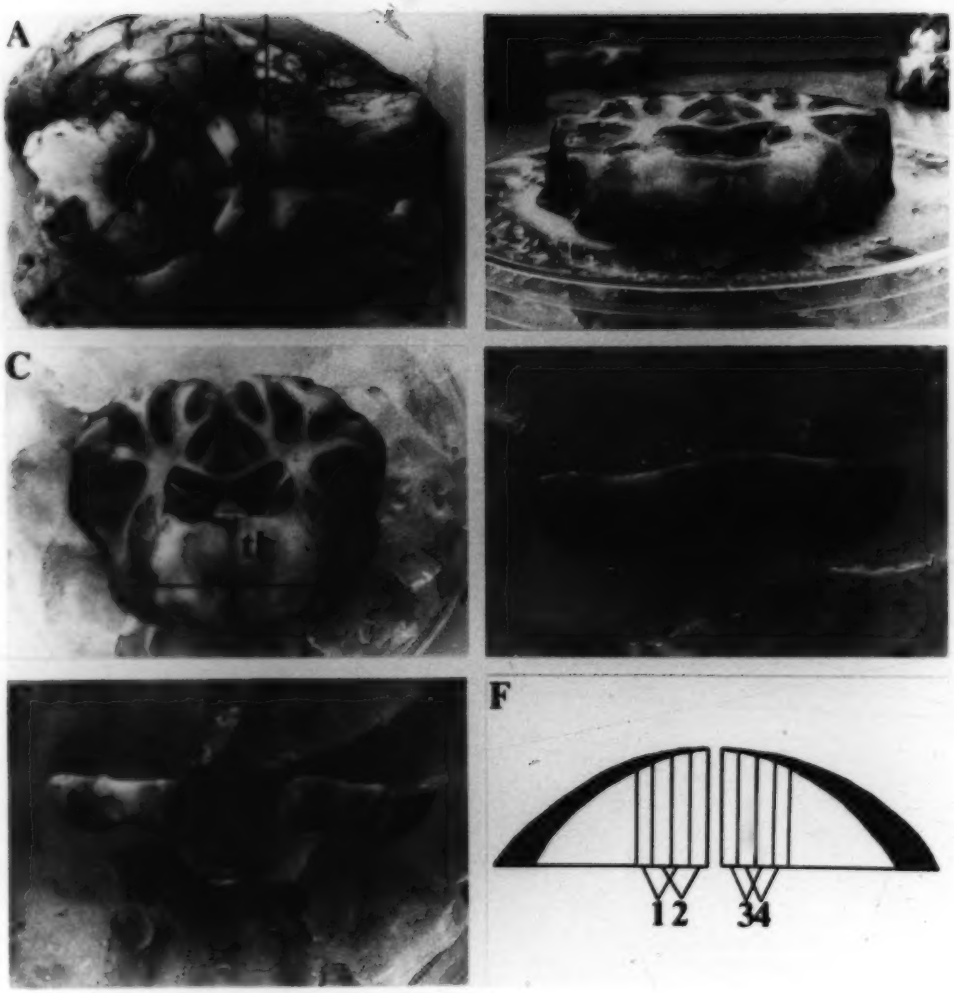
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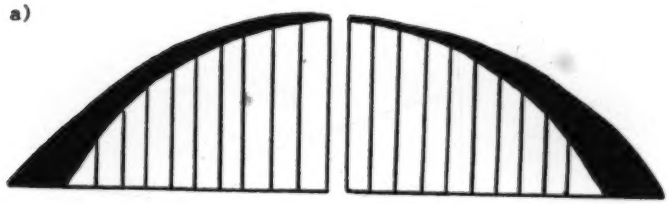
FIGURE A.2 Schematic representation of hypothalamic slicing techniques. a) initial slicing technique for Parts 1, 2 and 3 of the preliminary studies, when entire sliced (500μm slices) halves were included in the perfusion. b) slicing technique used in Part 4, hypothalamic halves vs quarters. Shaded slices on the right represent one quarter, and white slices on the right represent the remaining quarter. c) slicing technique used in Part 5, regional distribution of GnRH secretory potential.

a)



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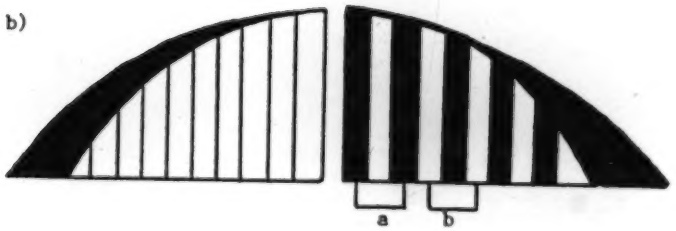

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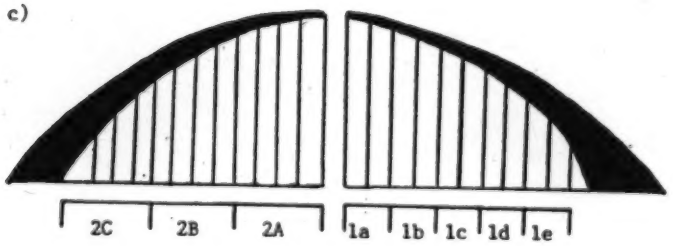
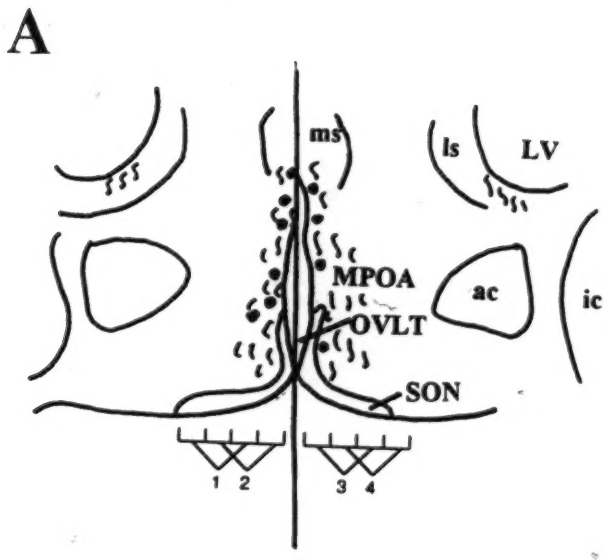


FIGURE A.3 Schematic representation of the hypothalamic nuclei assumed to be included in the tissue slices included in the perfusion chambers. Solid circles indicate GnRH perikarya and fine lines indicate GnRH processes. Schematics A through D represent rostral to caudal coronal slices through the hypothalamus. The bars in each diagram represents the sagittal slices used in the perfusion and the number of chambers represented by these slices. 3V = third ventricle; ac = anterior commissure; AH = anterior hypothalamus; ARC = arcuate nucleus; fx = fornix; ic = internal capsule; ls = lateral septal area; LV = lateral ventricle; ME = median eminence; MPOA = medial preoptic area; ms = medial septal area; OC = optic chiasm; OT = optic tract; OVLT = organum vasculosum of the lamina terminalis; PeV = periventricular nucleus; PVN = paraventricular nucleus; SON = supraoptic nucleus; VMN = ventromedial nucleus. Compiled and modified from Kineman *et al.* (1988) and Leshin *et al.* (1994).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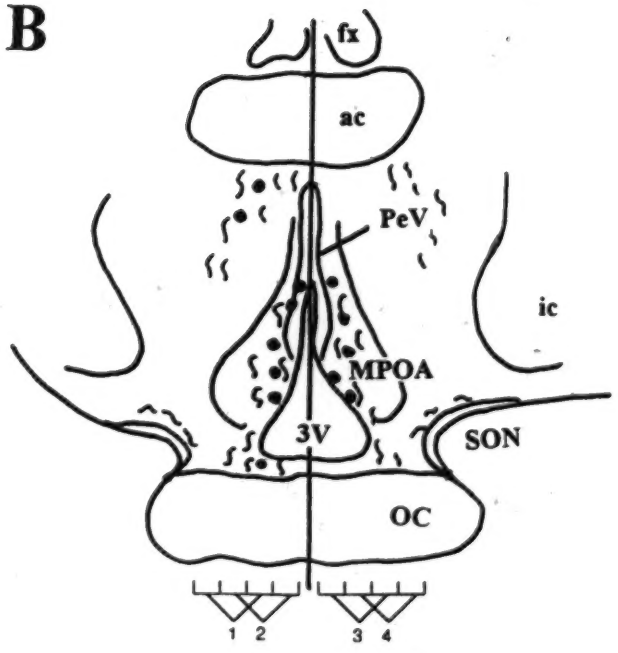
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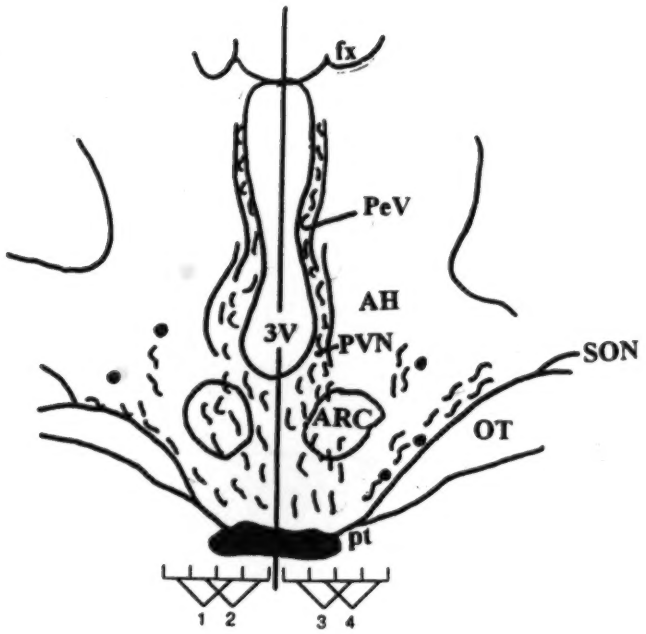
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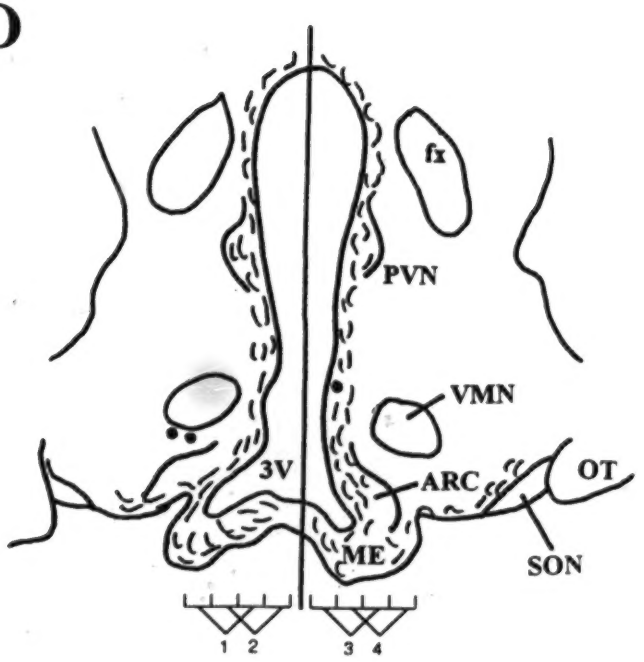
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1 2
3 4

FIGURE A.4 Changes in the sensitivity of the GnRH assay standard curve in response to changing: a) antibody dilutions from 1:80,000 to 1:120,000, 1:160,000 and 1:320,000; b) number of radioactive counts per minute per tube (cpm/tube) from 15,000 cpm/tube to 5,000, 10,000 and 20,000 cpm/tube; c) the antibody dilution and setting the number of cpm/tube at 5,000 cpm/tube. There was no increase in assay sensitivity in response to any of these assay changes.

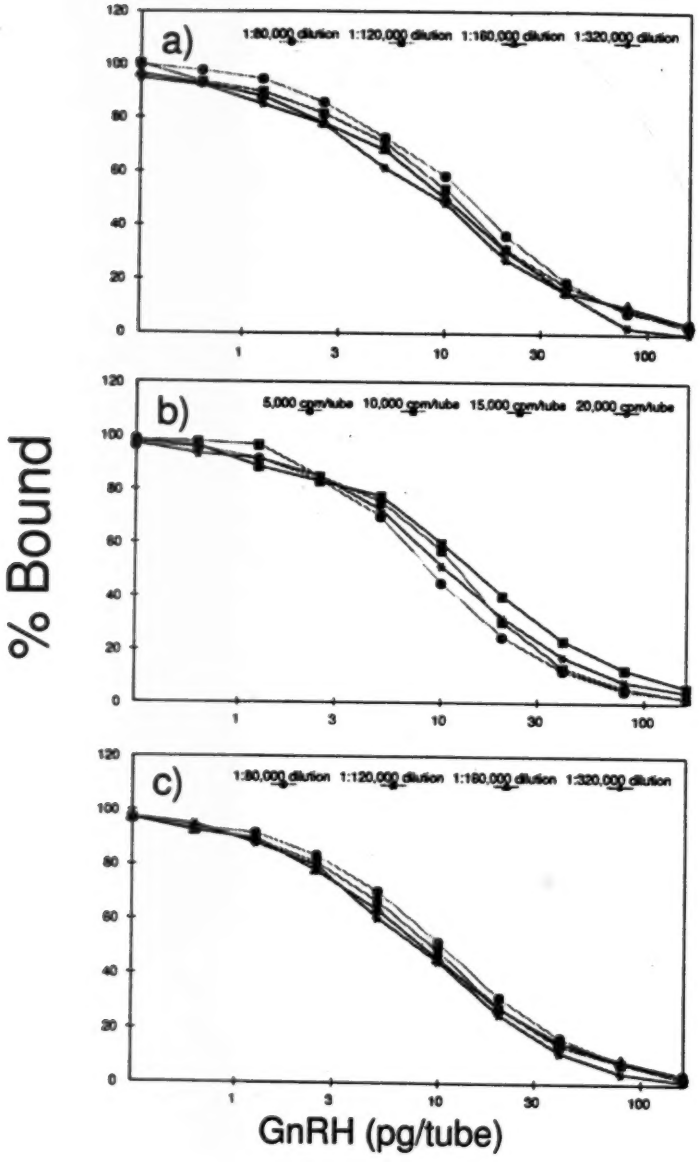
a)
1:80,000 dilution 1:120,000 dilution 1:160,000 dilution 1:320,000 dilution
b)
5,000 cpm/tube 10,000 cpm/tube 15,000 cpm/tube 20,000 cpm/tube
c)
1:80,000 dilution 1:120,000 dilution 1:160,000 dilution 1:320,000 dilution
% Bound
GnRH (pg/tube)

FIGURE A.5 a) Parallelism of pooled transport media (TM) and pooled previously concentrated-reconstituted TM, at 50, 100 and 200 µl volumes against the standard curve, and b) parallelism of TM at 12.5, 25, 50, 100 and 200 µl volumes against the standard curve. These are representative parallelisms from 2 different assays; pooled TM was run at volumes 50, 100 and 200 µl volumes in all assays as a control.

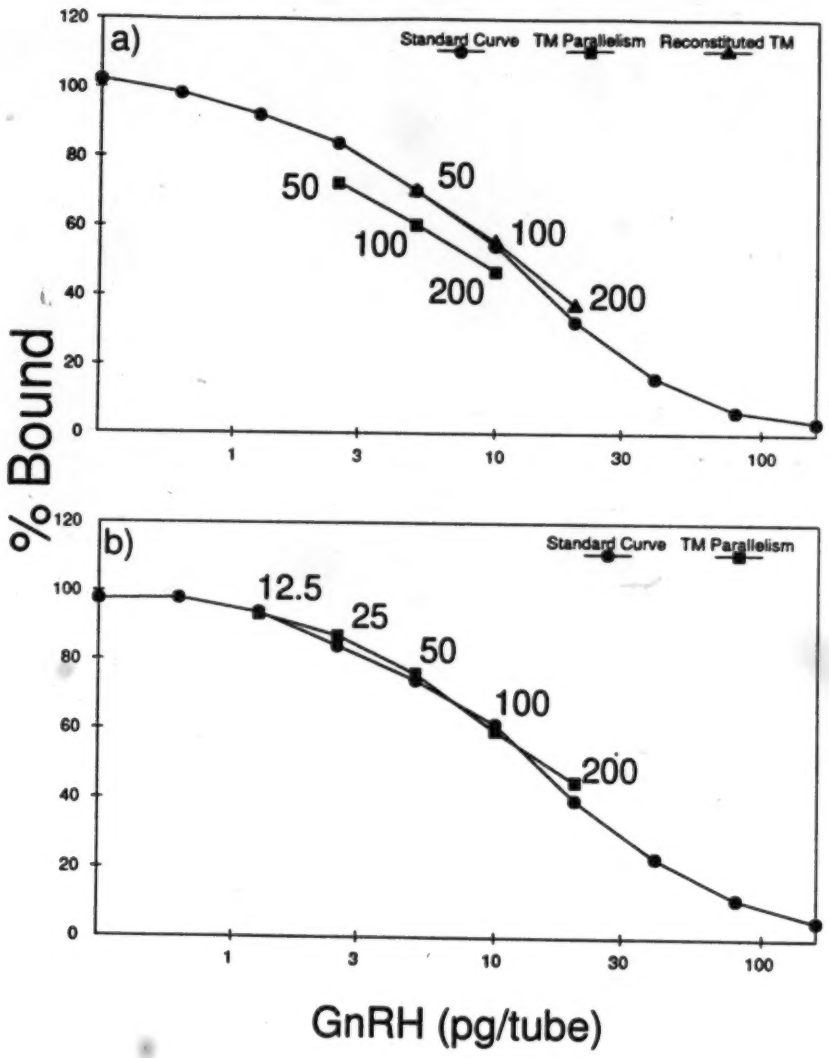
a)
Standard Curve
TM Parallelism
Reconstituted TM
50
50
100
100
200
200
b)
Standard Curve
TM Parallelism
12.5
25
50
100
200
% Bound
GnRH (pg/tube)
120
100
80
60
40
20
0
1
3
10
30
100

FIGURE A.6 a) Mean (- SEM) concentration of GnRH found in hypothalamic halves from the barrows in Parts 1, 2 and 3 of the preliminary studies. There was no difference between the two halves in either the leakage/secretion of GnRH into transport media (P = 0.26) or the remaining GnRH content in the hypothalamus following perfusion (P = 0.68). b) Mean (- SEM) concentration of GnRH found in hypothalamic halves and quarters from the barrows in Part 4 of the preliminary study. There was no difference between the halves or quarters in either the leakage/secretion of GnRH into transport media (P = 0.09) or the remaining GnRH content in the hypothalamus following perfusion (P = 0.74).

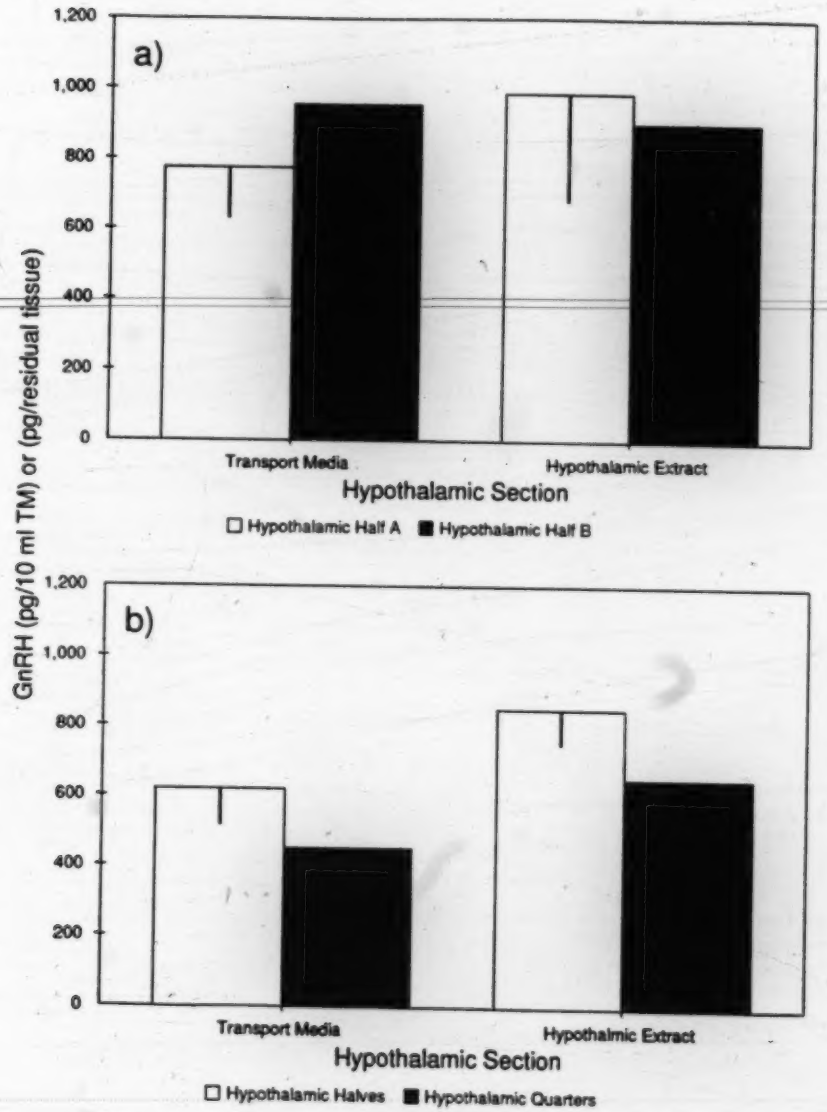
a)
1,200
1,000
800
600
400
200
0
Transport Media
Hypothalamic Extract
Hypothalamic Section
Hypothalamic Half A
Hypothalamic Half B
GnRH (pg/10 ml TM) or (pg/residual tissue)
b)
1,200
1,000
800
600
400
200
0
Transport Media
Hypothalmic Extract
Hypothalamic Section
Hypothalamic Halves
Hypothalamic Quarters

FIGURE A.7 *In vitro* perfusion GnRH profiles from MPOA/MBH tissue obtained from 2 market weight barrows in Part 4. Arrows indicate a 1 min depolarizing pulse of 1.38M KCl. Open circles denote GnRH profiles from hypothalamic halves and solid circles denote GnRH profiles from hypothalamic quarters.

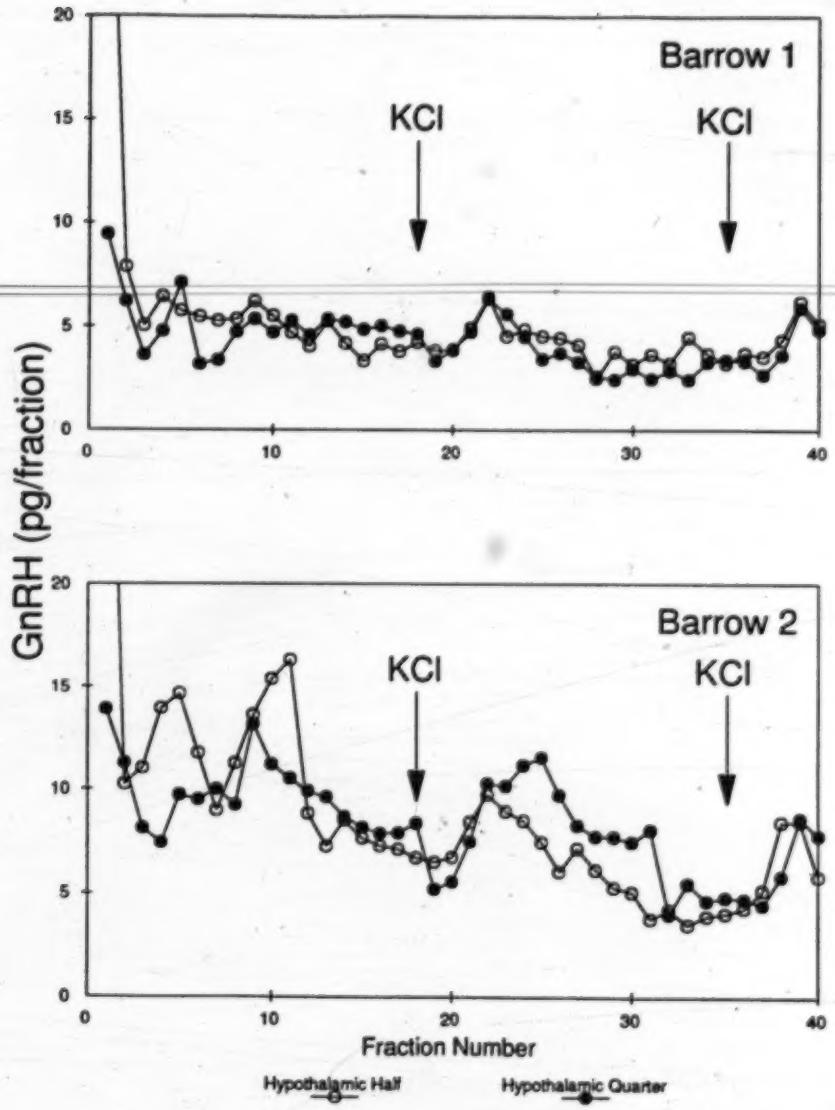
Barrow 1
KCl
KCl
Barrow 2
KCl
KCl
GnRH (pg/fraction)
Fraction Number
Hypothalamic Half
Hypothalamic Quarter

TABLE A.1 Time points of analysis across the perfusion of hypothalamic fragments sliced from different areas of the hypothalamus (see text and Figure A.2c for description) showing relative mean secretion between slices. Order of GnRH secretion from tissues does not take into account SEM and therefore there is a discrepancy at time point 9, where mean secretion from slice 1c is not significantly different from 1a, but secretion from slice 1d is greater than 1c (ie. 1c has a large SEM associated with mean GnRH secretion). *Stimulation with KCl at the beginning of these fractions, main response to stimulation occurs in the fraction following each of these fractions (ie. at time points 8 and 13).
^{x} differs from hypothalamic fragment 1a; ^{y} differs from hypothalamic fragment 2A ($P<0.05$).

Time Point During Analysis (15 min mean fraction)	Mean GnRH Secretion Relative to Other Tissue Slices Over Analysis Time Points	
time 1 (2-4)	1d >1c >1a >1b >1e	2B >2C >2A
time 2 (5-7)	1c >1a >1d >1b >1e	2A >2B >2C
time 3 (8-10)	1a >1d >1c >1b >1e	2A >2B >2C
time 4 (11-13)	1a >1c >1b >1d >1e	2A >2C >2B
time 5 (14-16)	1a >1b >1c >1d >1e	2A >2C >2B
time 6 (17-19)	1a >1b >1c >1d >1e	2A >2C >2B
time 7 (20-22)*	1a >1b^{x} >1c^{x} >1d^{x} >1e^{x}	2A >2B^{y}>2C^{y}
time 8 (23-25)	1a >1c >1b^{x} >1e^{x} >1d^{x}	2A >2C^{y} >2B^{y}
time 9 (26-28)	1a >1d^{x}>1c >1e^{x} >1b^{x}	2A >2C^{y} >2B^{y}
time 10 (29-31)	1a >1e >1c >1d^{x} >1b^{x}	2A >2C^{y} >2B^{y}
time 11 (32-34)	1a >1e >1d >1c >1b	2A >2C^{y} >2B^{y}
time 12 (35-37)*	1a >1c >1e >1d >1b	2A >2C^{y} >2B^{y}
time 13 (38-40)	1a >1b >1c >1d^{x} >1e^{x}	2A >2C^{y} >2B^{y}